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The Role of HER-Targeted Tyrosine Kinase Inhibitors in the Treatment of High-Grade Serous Ovarian Cancer

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Declaration

I declare I am the author of this dissertation. This work is entirely my own, unless otherwise stated, where due credit to the contributor is given. No part of this work has been submitted for any other degree or professional qualification.

Maria Bonello

Abstract

High-grade serous ovarian cancer (HGSOC) has the highest incidence rate of the various subtypes of ovarian cancer. HGSOC patients usually respond to initial platinum therapy, however approximately 70% of patients relapse, or worse, become resistant to therapy. Members of the Human Epidermal growth factor Receptor (HER) family, especially EGFR and HER2, are frequently involved in disease progression, hence strategies to inhibit their action could prove advantageous as treatment for selected ovarian cancer patients. Monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs) targeted against the HER family have demonstrated valuable anti-tumour activity in multiple other cancer types, and their possible use in ovarian cancer merits further study.

This study sought to evaluate the effectiveness of HER-targeted therapy in platinum-resistant and platinum-sensitive HGSOC, and the implications of HER family expression and other biomarkers in response to treatment. The effects of five TKIs (afatinib, canertinib, lapatinib, neratinib and sapitinib) targeted against multiple HER family members on cellular functionality were studied in three pairs of HGSOC cell lines, wherein each pair was derived from the same patient before and after clinical resistance to platinum-based chemotherapy. The effects of the TKIs were compared with the anti-HER2 mAbs trastuzumab and pertuzumab. The outcomes of modulation of EGFR, HER2 and HER3 expression on treatment sensitivity were also investigated, along with gene expression differences upon treatment.

The TKIs were found to be effective in inhibiting proliferation, migration and invasion, even in the presence of epidermal growth factor (EGF) and heregulin (HRG). The mAbs investigated were not as effective as the TKIs. Combination strategies of TKI with cytotoxic agents (cisplatin, carboplatin and paclitaxel) and TKI with mAbs were compared for their anti-proliferative behaviour. Combinations involving neratinib with pertuzumab or cisplatin demonstrated a degree of synergy. Knock-down of EGFR expression affected the anti-migratory effects of neratinib, but did not influence its anti-proliferative activity. Overexpression of HER2 or HER3 in the platinum-sensitive cell lines caused these cells to become more sensitive to TKIs, whilst the contrary

happened when HER2 or HER3 were overexpressed in the platinum-resistant cell lines.

The MAPK pathway was stimulated by EGF and HRG, as was the PI3K pathway. Protein expression through western blot analysis showed that neratinib decreased phosphorylation of ERK and Akt in the cell lines. Neratinib was the most potent TKI of those tested in growth inhibition studies, and was used to investigate the effects of TKI treatment on gene expression. Neratinib down-regulated the MAPK and PI3K pathways in most cell lines, even in the presence of HRG, whilst reducing proliferation and migration processes. Based on gene expression data, CCAAT Enhancer Binding Protein Gamma (CEBPG), DNA Damage Inducible Transcript 4 Like (DDIT4L), Ral guanine nucleotide dissociation stimulator (RALGDS), and Sprouty homolog 2 (SPRY2) were identified as possible HER-targeted therapy-induced biomarkers, whose expression changed upon neratinib-treatment from this data set, and mAb-treatment based in another data set. Assessment of these proteins in tissue microarrays consisting of ovarian cancer xenografts treated with mAbs over a period of days, identified higher expression in the mAb-treatment group than the controls for all proteins except SPRY2, which had lower expression.

In conclusion, treatment with HER-targeted TKIs could be a useful approach even in the treatment of platinum-resistant HGSOC, where the expression of EGFR, HER2 and HER3 play an important role in determining TKI sensitivity.

Lay Abstract

Imagine your garden with plants and trees, and then unwanted weeds start to grow. Ovarian cancer is the weed, and is the 8th most deadly cancer type in women worldwide. Current treatments for ovarian cancer, which include removing as much as possible of the cancer and administering platinum-based chemotherapy is effective in about 70% patients, but as with using herbicides in a garden, some cancers/weeds become resistant and continue to grow. Therefore, this project aimed to study the effects of other therapies (herbicides) that target ovarian cancer (weed) more specifically. The novel treatments act upon one or more of a family of four molecules, known as HER1, HER2, HER3 and HER4.

This study found that the targeted therapies evaluated (called afatinib, canertinib, lapatinib, neratinib and sapitinib), were more able to inhibit growth, migration and invasion in the ovarian cancer cells that were resistant to initial therapy, than in the ovarian cancer cells that were sensitive to initial therapy. This was all achieved at drug concentrations that humans can safely tolerate. The combination of one of the above targeted therapies (neratinib or lapatinib) with platinum-based drugs or pertuzumab (another targeted therapy), produced results that were much more effective than single therapy alone. Altering the level of the target molecules (HER1, HER2 and HER3) changed the response to treatment.

A large data-driven experiment to examine changes induced by one of these drugs, neratinib, identified a number of key genes, which like the characteristics of weeds responding to a new herbicide could be used to identify how and which patients will respond to new treatments.

In conclusion, these new drugs were effective in ovarian cancer cells, and therefore could be a promising treatment option to patients who stop responding to first-line ovarian cancer therapy.

Publication

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List of Abbreviations

ABC	ATP-binding cassette transporter	**IC_{50}**	Half maximal inhibitory concentration
Akt	Protein kinase-B	**mAb**	Monoclonal antibody
APS	Ammonium persulfate	**MAPK**	Mitogen-activated protein kinase
ANOVA	Analysis of variance	**min**	minute
BCA	Bicinchoninic acid	**mTOR**	Mechanistic target of rapamycin
BRCA	Breast cancer gene family	**NCBI**	National Centre for Biotechnology Information
CA125	Cancer antigen 125	**NICE**	The National Institute for Health and Care Excellence
CEBPG	CCAAT enhancer binding protein γ	**OD**	Optical density
CI	Combination index	**OS**	Overall survival
CO_2	Carbon dioxide	**p-**	Phosphorylated
DAB	3,3'-Diaminobenzidine	**P/S**	Penicillin/streptomycin
DAPI	4',6-diamidino-2-phenylindole	**PAGE**	Polyacrylamide gel electrophoresis
DAVID	Database for Annotation, Visualization & Integrated Discovery	**PARP**	Poly-ADP-ribose polymerase
DDIT4L	DNA damage inducible transcript 4 like	**PBS**	Phosphate buffered saline
DMSO	Dimethylsulfoxide	**PBST**	Phosphate buffered saline tween20
DNA	Deoxyribonucleic acid	**PFS**	Progression free survival
ECM	Extracellular membrane	**PI3K**	Phosphatidylinositol 3 kinase
EGF	Epidermal growth factor	**Pt**	Platinum
EGFR	Epidermal growth factor receptor	**RALGDS**	Ral guanine nucleotide dissociation stimulator
EMT	Epithelial–mesenchymal transition	**RB**	Retinoblastoma
ERK	Extracellular signal-regulated kinase	**RNA**	Ribonucleic acid
FBS	Foetal bovine serum	**RPMI**	Roswell Park Memorial Institute medium
FDA	Food and Drug Administration	**SDS-PAGE**	Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis
FDR	False discovery rate	**SEM**	Standard error of the mean
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique	**siRNA**	Small interfering ribonucleic acid
GEO	Gene Expression Omnibus	**SPRY2**	Sprouty homolog 2
GO	Gene Ontology	**SRB**	Sulforhodamine B
h	hour	**TEMED**	Tetramethylethylenediamine
HER	Human epidermal growth factor receptor	**TKI**	Tyrosine kinase inhibitor
HGSOC	High-grade serous ovarian cancer	**TMA**	Tissue microarray
HRG	Heregulin	**TP53**	Tumour protein 53
IHC	Immunohistochemistry	**WT**	Wild type
IL	Interleukin	**µM**	Micromolar

1 Introduction

Cancer is characterised as a group of diseases that proliferate abnormally and have the ability to invade or metastasise from their origin to other parts of the body. There were approximately 18 million new cancer cases worldwide and around nine million fatalities due to cancer in 2018, of which almost nine million new incidences and around four million deaths occurred in women [1]. These incidence rates are predicted to be reducible by about 40% in high-income countries, by decreasing exposure to environmental risks and changing lifestyle [2]. In males, lung cancer followed by prostate cancer are the most common types of cancer. In women, breast cancer, followed by colorectal cancer and lung cancer are the most frequently occurring [1]. Ovarian cancer is the 8th most common cancer type in females, bearing 3.4% of the total female cancer incidence rate (Figure 1.1) [1]. Although worldwide cervical cancer still kills more women than ovarian cancer, in most Western countries, ovarian cancer has become the most lethal gynaecological malignancy. This is because cervical cancer is frequently detected at an earlier stage through screening and is also prevented (to a large degree) by HPV vaccination [1, 3].

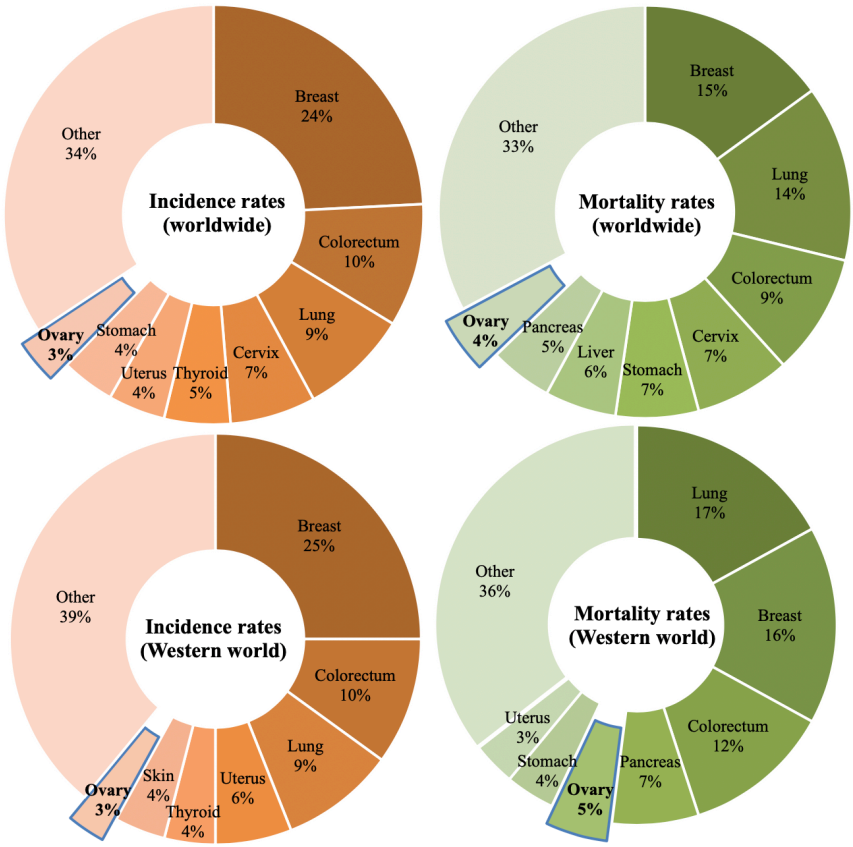


Figure 1.1: Incidence and mortality rates of cancer types in women in 2018

Pie charts represent the most common cancer types in women worldwide versus in the Western world. Adapted from [1].

1.1 Hallmarks of cancer

The hallmarks of cancer encompass six biological traits that cancer cells, including ovarian cancer cells, acquire during the development of tumours. This notion was developed by Hanahan and Weinberg in 2000 [4], and revised in 2011 [5]. These hallmarks include the following:

Sustained proliferative signalling: Whilst normal cells regulate the activity of growth-promoting signals, cancer cells are frequently unable to control these signals [5]. This leads to the over-activity of molecules such as tyrosine kinases and downstream signalling pathways, notably the MAPK and PI3K pathways in ovarian cancer [6] (discussed in detail in section 1.4). Therefore, in ovarian cancer, targeted inhibitors that target tyrosine kinases and other molecules up-stream of the MAPK and PI3K pathways would be beneficial in blocking the initiation of these signals, leading to uncontrolled proliferation (discussed further in section 1.6).

Activation of invasion and metastasis: Cancers migrate from the primary site to other body sites due to their ability to invade neighbouring tissue and metastasise in a multi-step process. In ovarian cancer, cells which detach from the primary tumour are carried by the physiological movement of the peritoneal fluid and adhere to the mesothelial cells mediated by integrins [7]. Once bound to the mesothelial cells, the tumour cells can invade the peritoneum and omentum [8].

Evasion of growth suppressors: Cancer cells are able to circumvent the natural negative regulation of cell proliferation, through tumour suppressors such as RB (retinoblastoma-associated) protein and TP53 protein [5]. In ovarian cancer, some patients present with alterations to the RB pathway and mutations in TP53 [9] (discussed further in section 1.3.1).

Induction of angiogenesis: Cancers have the ability to generate neovasculature in order to sustain the tumour with nutrients and oxygen, and eliminate waste components. Key proteins that sustain angiogenesis are vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1) [5]. In ovarian cancer, bevacizumab, which inhibits VEGF-A, was the first anti-angiogenic agent to be approved for the management of recurrent ovarian cancer [10].

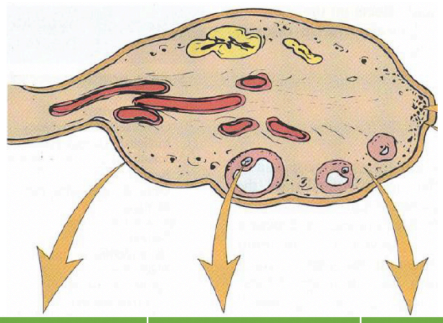
Resistance to cell death: Programmed cell death serves as a natural process to stop cancer initiation. However, abnormalities in regulators such as TP53 trigger resistance to cell death. In ovarian cancer the Bcl-2 family plays an important role in pro-apoptotic and anti-apoptotic mechanisms [11].

Other factors which are associated with cancer survival and development include **tumour-promoting inflammation**, which also helps with the formation of new blood vessels. Their **genome instability and ability to mutate**, even to treatment, makes tumours harder to treat, along with **replicative immortality**, meaning that they are capable of indefinite growth. Emerging hallmarks include capability to **avoid immune interaction** to circumvent destruction as well as **deregulating cellular energetics**, whereby tumours adjust their metabolism to enable their uncontrolled growth [5].

1.2 Ovarian cancer overview

1.2.1 Aetiology

The most common form of ovarian cancer are carcinomas (malignant epithelial tumours) representing over 90% of cases [12]. Other types of ovarian cancer include the sex cord-stromal tumour (eg granulosa cell tumours), which occurs in 5-6% of tumours and germ cell tumours (eg yolk sac tumours and dysgerminomas), which occur in 2-3% of cases [12] (Figure 1.2). Recent understanding of the pathology of ovarian cancer has revealed that ovarian cancer cells frequently originate from sites other than the ovaries, such as the fallopian tubes and involve the ovaries secondarily [13, 14]. Although this is a speculative hypothesis, it is supported by the fact that the histology of serous ovarian cancer is similar to the morphology and TP53 signatures of the fallopian tube [15], suggesting that the neoplastic process could initiate at the tubal lesions and migrate to the ovaries where they progress aggressively. Ovarian endometriosis, occurring through retrograde menstruation, is also thought to give rise to other subtypes (endometrioid and clear cell) of ovarian cancer [16, 17].



Form	Epithelial tumours	Sex cord stromal tumours	Germ Cell tumours
Frequency (%)	90	5-6	2-3
Types	• High-grade serous • Endometrioid • Mucinous • Clear Cell • Low-grade serous	• Pure sex cord (Granulosa cell tumours) • Pure stromal (Fibroma, thecoma) • Mixed (sertoli-Leydig cell tumour)	• Yolk sac tumours • Dysgerminomas

Figure 1.2: Aetiology of ovarian cancer

The common forms of ovarian cancer and their frequency detailed in this diagram [1] [18].

[1] Krafts K. Presentation: Female Reproductive System. https://slideplayer.com/slide/12109617/. Accessed on 03/04/2019.

1.2.2 Risk factors and epidemiology

The incidence rate of ovarian cancer is highest in North America and Europe [19], and the lifetime risk of having ovarian cancer stands at around 1.5% [20]. Risk factors for developing ovarian cancer include both genetic and hormonal elements. The risk increases by three to seven-fold if a woman has first-degree family members who developed ovarian cancer [21]. Germline mutations in *BRCA1* or *BRCA2* increase the lifetime risk of developing ovarian cancer by 44% and 27% respectively [22-24], whilst Lynch syndrome, which is characterised by mutations in the mismatch repair system, increases the risk of ovarian cancer by fourteen-fold [25, 26]. Other predisposing risk factors include increasing age, infertility, endometriosis, hormonal factors (such as late age at menopause, hormone replacement therapy and early age at menarche) and obesity [27-29]. On the other hand, factors that decrease the risk of ovarian cancer include pregnancies, breast feeding, oral contraceptive pill and hysterectomy [28-32].

1.2.3 Screening, diagnosis and staging

Ovarian cancer patients usually present with non-specific symptoms, which contributes to the low percentage (approximately 20%) of patients being diagnosed when at early stages of the disease. Symptoms include abdominal discomfort, pelvic pain, loss of appetite, weight loss, frequency in urination, changes in bowel habits and ascites [33]. A palpable pelvic mass can sometimes be found on clinical examination. Women are not offered routine screening, however when there is a suspicion of ovarian cancer, the National Institute for Health and Care Excellence (NICE) recommends initial investigations using ultrasonography and/or sequential blood tests for the biomarker Cancer Antigen 125 (CA125) [34]. The drawback of CA125 is that it is a non-specific marker in premenopausal females and can be increased during menstruation, fibroids, pelvic inflammatory disease or pregnancy. Additionally, about 20% of ovarian cancers do not express this antigen, while this test is less sensitive in patients presenting at early stages of ovarian cancer [35]. However, a recent study- UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS)- assessing the effect of screening with multimodal screening (using CA125 with risk of ovarian cancer

algorithm) or transvaginal ultrasound screening found that multimodal screening helped reduce mortality when prevalent cases were excluded [36].

Recent studies have tried to look at other potential biomarkers for early ovarian cancer diagnosis. One of the biomarkers was HE4, which appears specific to ovarian carcinoma, and is expressed in about 34% of ovarian cancers which do not express CA125 [35]. However, when a Risk of Ovarian Malignancy Algorithm together with HE4 serum levels were assessed against CA125 performance alone, it emerged that CA125 was a better predictive marker of ovarian carcinoma [37]. Another potential biomarker is HK10, which was absent in normal cells, but was expressed in ovarian carcinoma, as well as in CA125-deficient cancers [35].

Staging of ovarian cancer is necessary for comparative terminology between oncology centres as well as to help develop the optimal clinical plan for each individual patient [12]. The FIGO (Fédération Internationale de Gynécologie et d'Obstétrique) staging of ovarian cancer is depicted in Figure 1.3. Imaging modalities, such as ultrasound, computerised tomography (CT), magnetic resonance imaging (MRI) and Positron emission tomography–computed tomography (PET-CT) scanning technologies are used to help stage ovarian cancer [38].

The approximate five-year survival rate stands at 47% [39, 40]. This survival rate depends on the stage at diagnosis. The five-year survival rate for patients diagnosed at early stages of ovarian cancer is 93%, while patients diagnosed in stage III or IV have a five-year survival rate of approximately 42% and 26% respectively [40].

Figure 1.3: Pictorial depiction of FIGO staging

The four stages of ovarian cancer and what is clinically defined by each stage, based on [12]. The red circles indicate tumour position.

1.2.4 Management and treatment

Ovarian cancer management is largely based on the stage at which the patient presents. The current first-line treatment regimen for patients with advanced ovarian cancer consists of tumour surgical debulking, combined with adjuvant therapy consisting of carboplatin with or without paclitaxel for six cycles (Figure 1.4) [41]. The response rate to first line management of advanced ovarian cancer is at 70-80% [42]. Some late stage ovarian cancer patients are also offered neo-adjuvant therapy, to improve chances of successful tumour debulking.

Combination of platinum-based therapy with paclitaxel has not been without some controversy, as clinical studies have shown differing results. A GOG-123 (Gynaecologic Ovarian Group #123) clinical trial assessing the overall benefit from the combination versus monotherapy showed that cisplatin alone or in combination with paclitaxel produced better progression free survival (PFS) than paclitaxel alone [43]. In fact the PFS was 16.4, 10.8 and 14.1 months for cisplatin alone, paclitaxel alone and the combination respectively [43]. However, the cisplatin dose when used as monotherapy was higher than the dose used in combination with paclitaxel, which led to more side effects [43]. Other earlier clinical studies, the GOG-111 and OV10 (European-Canadian Intergroup trial) which compared cisplatin and paclitaxel versus cisplatin and cyclophosphamide, have shown that the PFS was over 15.5 months versus 11.5 months respectively, while overall survival (OS) was over 10-14 months longer in patients receiving paclitaxel [44, 45]. Clinical studies evaluating the role of carboplatin in combination with paclitaxel, showed that this combination was better tolerated than cisplatin plus paclitaxel. However, both treatment arms had similar PFS and OS of around 18 months and 44 months respectively [46].

Bevacizumab, which is an anti-VEGF agent, is currently marketed for the management of advanced ovarian cancer in combination with carboplatin and paclitaxel [47]. It is also used in combination with carboplatin plus gemcitabine or paclitaxel in primary recurrent platinum-sensitive ovarian cancer. In platinum-resistant recurrent ovarian cancer, it is used in addition to paclitaxel, topotecan or pegylated liposomal doxorubicin [47]. Recent FDA-approved drugs for the maintenance treatment of

patients with platinum-sensitive relapsed *BRCA*-mutated ovarian cancer (HGSOC) are the poly-ADP ribose polymerase (PARP) inhibitors, olaparib and niraparib [48, 49].

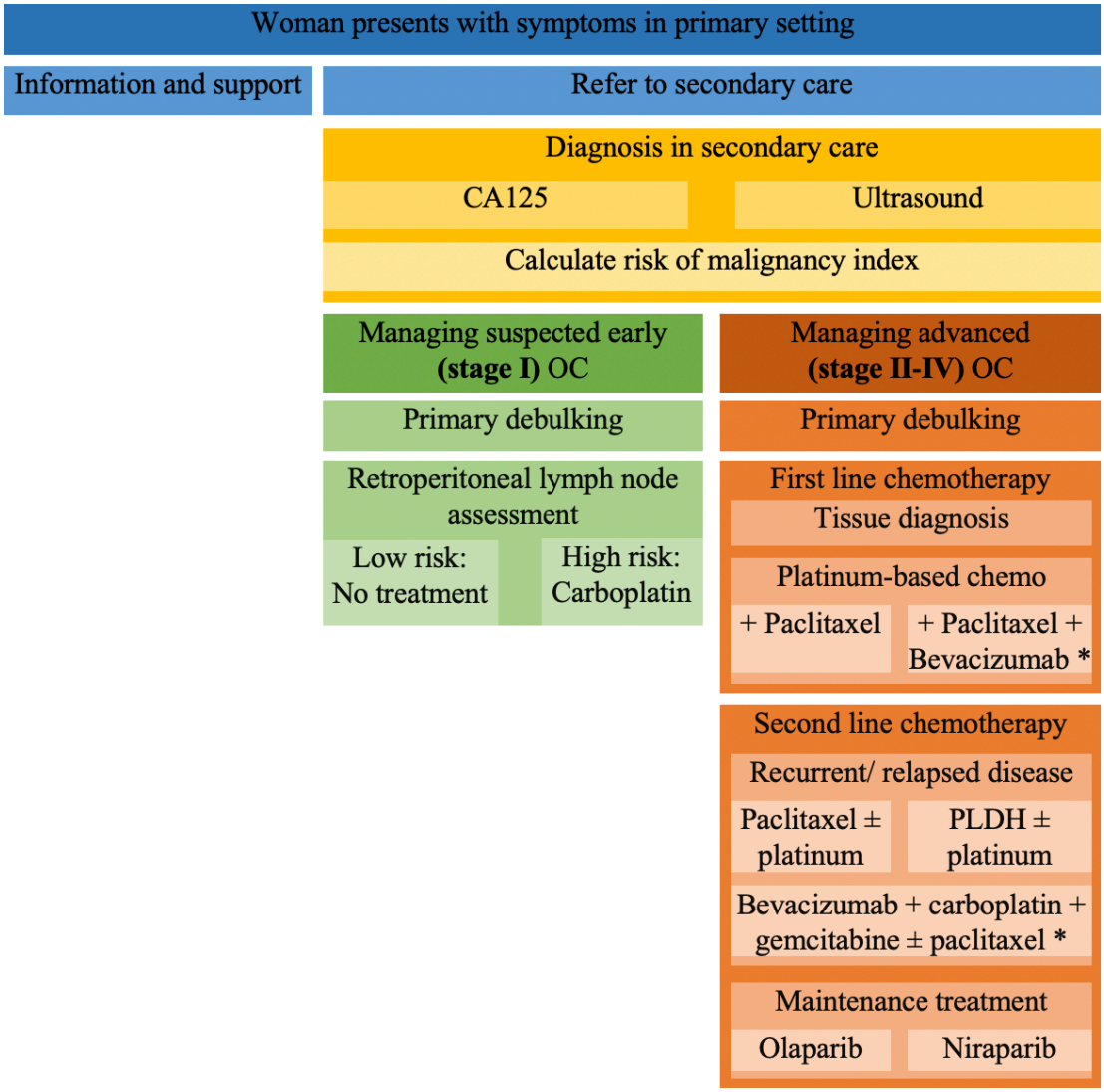


Figure 1.4: Summary of management and treatment of ovarian cancer

Information is based on NICE guidelines for ovarian cancer, updated in February 2019[2]. PLDH refers to Pegylated liposomal doxorubicin hydrochloride, and the asterisk (*) refers to treatments which although are within the indications of the product's marketing authorisation, they are not recommended to be used in the clinical setting by NICE.

[2] NICE. Ovarian Cancer Overview. First published in Feb 2012. Updated in Feb 2019. Accessed on 26/02/2019. Available from: https://pathways.nice.org.uk/pathways/ovarian-cancer.

1.2.4.1 Platinum-based chemotherapy

Platinum-based chemotherapy was discovered by serendipity in the 1960s, with an experiment to investigate electromagnetic fields on bacterial growth. When voltage was applied to these bacteria, they were observed to grow in filaments, over 300 times their normal length. This cell division interference was due to cis-diamminetetrachloroplatinum (cisplatin; CDDP) [50]. The first platinum-based agent used in ovarian cancer was cisplatin, which showed significantly longer survival rates in contrast to the alkylating agent used at the time, cyclophosphamide [51]. Later studies led to a safer second-generation agent, carboplatin which was approved as standard of care for the treatment of ovarian cancer in 1989 [52]. Carboplatin offers less nephrotoxicity, less toxicity in the gastrointestinal tract and is less neurotoxic than cisplatin [53].

Platinum-based chemotherapy is toxic to dividing cells, including cancer cells, as it forms interstrand and intrastrand DNA cross-links. While intrastrand cross-links occur in 90% of DNA cross-linking, interstrand cross-links are stronger [54]. Following this DNA-induced damage, DNA repair pathways decide the fate of the cells. These cross-links can lead to single-strand or double-strand DNA breaks [41]. In response to the severity of DNA breaks, the cells can either initiate DNA repair, or induce apoptosis.

Normally, single-strand DNA breaks are repaired through nucleotide excision repair, whereas double-strand DNA breaks are repaired through non-homologous end joining and homologous recombination. Nucleotide-excision repair refers to a process whereby large DNA adducts are removed as they would be distorting the double DNA helix. Non-homologous end joining is the process joining and resealing the two ends of a DNA double strand. On the other hand, homologous recombination is the copying of DNA sequence from an intact DNA molecule to fix replication deformities [55].

In platinum-treated cells, the primary process of intrastrand DNA repair pathway following platinum-based chemotherapy is nucleotide excision repair. This process identifies and removes large DNA adducts as they distort the double DNA helix. Cells lacking nucleotide excision repair proteins such as XPC, XPA and ERCC1 exhibit no repair activity in platinum-treated cells. Nucleotide excision repair genes have been

shown to be altered in about 8% of epithelial ovarian cancer and these alterations are associated with improved overall survival and progression-free survival compared with patients without nucleotide excision repair alterations [56]. Conversely, high levels of the ERCC1 protein, involved in the nucleotide excision repair process, have been linked with poor response to chemotherapy in ovarian cancer, and its mRNA levels increased in response to cisplatin-induced DNA damage [57].

Double-strand DNA breaks are repaired through non-homologous end joining and homologous recombination. Non-homologous end joining is a repair process that joins and reseals the two ends of a DNA double strand in a Ku70/Ku86-dependent process which can result in error-prone repair, due to addition or loss of bases, or to ligation of the wrong ends [57]. Homologous recombination is an alternative mechanism dealing with double-strand break repair. It is an error-free mechanism that uses the sister chromatid as a template to repair the double strand break during the S and G2 phases of the cell cycle [56]. On recognition of the double strand break, BRCA1 facilitates recruitment of a complex (the MRN complex) which in conjunction with other key molecules resect the DNA ends. When end resection occurs, double strand breaks can be further repaired through error-free homologous recombination DNA repair by the recruitment of BRCA2 and the effector RAD51 to construct nucleoprotein filaments that catalyse the recombination between the broken strand and its undamaged homologue [56, 58].

The Cancer Genome Atlas (TCGA) consortium has estimated that about 50% of HGSOC cases have homologous recombination-based repair defects [59]. Approximately 20% of these defects are related to germline or somatic BRCA1 or BRCA2 mutations [60, 61]. BRCA1 methylation also occurs in another 11% HGSOC while EMSY methylation and changes in other homologous recombination repair genes (e.g. Fanconi Anaemia genes) can account for another 10% of cases [62].

Cells deficient in homologous repair (e.g. by BRCA1 and BRCA2 mutations or other components of the homologous recombination repair pathway) are unable to repair DNA strand breaks efficiently. This defect permits sensitivity not only to the platinum drugs but also to the PARP inhibitors since the homologous recombination-deficient

cells rely on this for cellular repair [56]. Mutations in BRCA1 and BRCA2 (germline or somatic) are associated with better overall responses to standard of care platinum-based chemotherapy and longer progression-free survival relative to non-mutated BRCA1/2 cases [63, 64]. Consistent with homologous recombination deficiency being associated with platinum sensitivity, restoration of homologous repair proficiency can be associated with platinum resistance. In both cell line models and in clinical cancers, reversion mutations in BRCA1 and BRCA2 have been demonstrated, resulting in the re-acquisition of homologous repair proficiency and development of resistance to both platinum drugs and PARP inhibitors [56].

1.2.4.2 Taxane chemotherapy

The taxanes were originally derived in 1971 from the bark of a Pacific Yew tree, whereby paclitaxel was the main constituent [65]. Later on, docetaxel was developed, which is a semisynthetic derivative of *T. baccata* needles [66]. Paclitaxel was approved by the FDA in 1992 to treat women with epithelial ovarian cancer. This approval was based on positive clinical trials in ovarian cancer patients. A phase II paclitaxel clinical trial in ovarian cancer demonstrated a PFS of over 4 months, irrespective of platinum-sensitivity [67]. In metastatic ovarian cancer patients, the median survival was 27 months and 6 months for responders and non-responders respectively [68]. A similar phase II clinical trial resulted in 30% of metastatic patients responding to therapy for 3-15 months [69].

Paclitaxel monotherapy has not been very favourable in ovarian cancer patients, however, using it in combination with platinum-based chemotherapy allowed for a reduction in platinum dose, thus decreasing the side effects [43]. Paclitaxel monotherapy has also shown prolongation of PFS when it was used as continuation therapy after platinum or paclitaxel therapy response. In fact, a 12-month cycle follow-on treatment with paclitaxel resulted in PFS of 28 months [70].

Taxanes, are cytoskeletal targeting drugs, acting on tubulin. Microtubules are important in normal cells as they help maintain shape, with motility and signal transmission. Cancer cells have defects in mitotic spindle assembly, chromosome segregation and cell division [71]. At sub-nanomolar concentrations, paclitaxel

stabilises microtubule polymer and inhibits metaphase spindle configuration. The microtubules formed by paclitaxel are stable and dysfunctional. Thus, cells are unable to progress to mitosis, and undergo apoptosis [71]. Mechanisms of acquired resistance to taxanes include an impaired ability of tubulins to polymerise, which in turn would have a slow rate to assemble microtubules [72]. Another mechanism of resistance is amplification of drug efflux pumps [72].

Nanoparticle, albumin-bound paclitaxel (Nab-paclitaxel) has been developed after paclitaxel, and is currently being used in breast cancer, lung cancer and pancreatic cancer. The formulation of nab-paclitaxel is not solvent-based, and this reduces the toxicities associated with excipients in solvents [73]. Additionally, preclinical studies found that the uptake of nab-paclitaxel in tumours is higher than conventional paclitaxel [74]. A phase II clinical trial evaluating the use of nab-paclitaxel in platinum-resistant ovarian cancer resulted in an OS of 17.4 months [75].

1.2.4.3 Anti-VEGF therapy

Bevacizumab (Avastin®) was the first anti-VEGF monoclonal antibody to be approved for the treatment of epithelial ovarian cancer, in combination with chemotherapy in advanced or recurrent cancer patients. It specifically targets VEGF-A, an isoform of VEGF, related to endothelial cell proliferation and migration. Bevacizumab is thought to cause regression in existing microvessels and inhibition of new vessel growth and neovascularisation (angiogenesis) [76].

Bevacizumab is a popular maintenance therapy, as a phase III clinical trial in newly diagnosed, late stage ovarian cancer patients (GOG-0218) has shown that patients who were administered bevacizumab throughout their therapy had their median PFS prolonged by four months over patients who received chemotherapy only [77]. Another similar phase III trial (ICON7) has shown a median OS of 34 and 39 months respectively in standard chemotherapy alone, versus addition of bevacizumab to standard chemotherapy, with beneficial results also observed in patients at high risk for disease progression [78]. More positive results were encountered when a phase III trial in platinum-resistant ovarian cancer patients, AURELIA, showed an improved PFS and OS by 3.3 months over chemotherapy alone in platinum-resistant ovarian

cancer patients [79]. A phase II clinical trial assessing the use of bevacizumab in combination with nab-paclitaxel in platinum-resistant ovarian cancer patients, had positive outcomes, whereby the PFS was approximately 8 months, while the OS was 17 months [80].

1.2.4.4 PARP inhibitors

PARP inhibitors inhibit the PARP enzyme, which is involved in the detection and initiation of DNA repair in response to single-strand DNA breaks [81]. PARP inhibitors induce replication fork collapse, which is frequently more pronounced in cancer cells due to their constant replication stress. These inhibitors are useful in the treatment of ovarian cancer, given that many ovarian cancer cases present with homologous recombination defects; as such, platinum sensitivity might be a marker for the treatment response to PARP inhibitors.

There are currently three PARP inhibitors approved for the maintenance treatment of platinum-sensitive relapsed ovarian cancer patients, with germline mutations in *BRCA*; olaparib (Lynparza®), niraparib (Zejula®) and rucaparib (Rubraca®). A phase III clinical trial in BRCA positive recurrent ovarian cancer patients, assessed the activity of olaparib versus current chemotherapy treatments (paclitaxel, gemcitabine topotecan or pegylated liposomal doxorubicin). It had positive results, whereby the olaparib treatment arm had a PFS of 13.4 months, versus 8.5 months in the current chemotherapy arm [82]. Pivotal clinical trials using of the PARP inhibitors include the olaparib SOLO-2 study, which was restricted to patients with *BRCA* mutations, and results showed that the quality adjusted PFS was over 6 months more in the olaparib-treated cohort than the placebo [83].

In the NOVA clinical trial assessing niraparib versus placebo in patients with or without *BRCA* mutations, the outcomes revealed that although in the mutated BRCA patients the PFS was greater, patients with wild type (WT) BRCA can also benefit from PARP inhibitors [84, 85]. Similarly, in the ARIEL-3 clinical trial assessing rucaparib's efficacy in *BRCA* mutated or non-mutated patients, although the PFS in BRCA mutated patients was increased, WT *BRCA* patients also benefitted from

therapy. The PFS difference in patients receiving PARP inhibitor versus placebo was 12 months in *BRCA* mutated patients, and 9 months in WT *BRCA* patients [86].

1.2.5 Platinum-refractory ovarian cancer

The time taken to relapse after initial platinum-based therapy is a positive indicator of the response to secondary platinum treatment. If the time to relapse is more than 12 months, there is a high chance for secondary platinum treatment sensitivity, while relapse within 6 months generally indicates platinum resistance [62]. Approximately 50% of relapsed patients have platinum-sensitive disease, whereas about 30% have platinum-resistant disease. The remaining 20% of patients termed to have platinum-refractory disease, are those who do not respond to platinum-therapy or progress whilst on treatment [87].

There are several plausible mechanisms to explain recurrent platinum-sensitive ovarian cancer. One of the hypotheses is the role of putative cancer stem cells, whereby the bulk of the tumour responds to chemotherapy, and small populations of chemotherapy-sensitive cells remain dormant, which start replicating upon treatment completion [88]. In addition, cells may become dormant due to the unfavourable microenvironment, such as in hypoxia or nutrient stress [89], and revive after some time. Platinum-sensitive ovarian cancer patients are managed by tumour debulking, followed by platinum-based chemotherapy and paclitaxel, with or without bevacizumab and maintenance therapy using PARP inhibitors. Other common regimens include carboplatin with gemcitabine and carboplatin with pegylated liposomal doxorubicin. Primary recurrent patients are still able to respond to therapy, with an improvement in PFS (29 months) and OS (13 months) [90]. A second or further relapse, is associated with a PFS of 5-12 months, with an OS of around 9 months [91, 92]. Optimal debulking plays an important role in the PFS and OS [92]. The PFS of patients having more relapses is similar to patients presenting with platinum-resistant disease, with response rates to therapy of only 10-20% [92]. There are no guidelines to platinum-sensitive ovarian cancer treatment beyond second recurrence, and therapy depends on the patient's ability to tolerate further cytotoxic agents.

Resistance frequently develops in patients who are initially sensitive to platinum-based therapy. Resistance to platinum-based chemotherapy in ovarian cancers could be due to inadequate drug exposure in first line therapy, for example due to insufficient dosing or poor distribution of treatment at tumour site. Reduced uptake of cisplatin into the cell due to alterations in trans-membrane transport systems is also detrimental [41] and studies show that platinum-resistant cell lines had 23-55% less cisplatin intake than sensitive cell lines [93]. Increased drug efflux by the multidrug-resistance associated proteins encoded by ABC genes, also plays an important role in chemotherapy resistance [94]. Amplification of 19q12 involving CCNE1 (occurs in about 20% of ovarian cancer patients, and intervenes in cell cycle progression and proliferation) is also associated with acquired primary treatment failure [95]. Resistance to therapy can also be determined by homologous recombination and other DNA damage repair pathways, whereby BRCA mutations are reversed [96, 97]. Epigenetic mechanisms, (defined by heritable changes to the genetic expression through mRNA regulation, histone modification and DNA methylation), are thought to play a crucial role in platinum-resistant disease [98].

Patients presenting with recurrent platinum-resistant ovarian cancer are rarely treated with a curative intent, and treatment is usually focused on controlling the symptoms and improving the quality of life. Clinical studies have looked at the possible use of pegylated liposomal doxorubicin versus gemcitabine in platinum-resistant ovarian cancer patients. Both monotherapies have resulted in a PFS of approximately 3.4 months, and an OS of 12.7 versus 13.5 months in gemcitabine and pegylated liposomal doxorubicin respectively [99]. Currently, there is no highly effective therapy for patients presenting with resistant disease, but anthracyclines (eg pegylated liposomal doxorubicin), docetaxel, and paclitaxel with or without bevacizumab are the most commonly administered therapies. These treatments generally have limited efficacy [100].

1.3 Classification of ovarian cancer

Traditional morphological description has classified epithelial ovarian cancer into five major groups- namely, serous, endometrioid, clear cell, mucinous and undifferentiated

cancers. In 2004, molecular analysis of ovarian cancer led to their classification into Type I and Type II cancers [101]. Type I tumours comprise low-grade serous, low-grade endometrioid, clear cell and mucinous types and often possess mutations in *HER2, BRAF, PTEN, CTNNB1, KRAS, ARID1A* and *PIK3CA* [17]. Low-grade tumours are more differentiated, tend to grow slowly and are less likely to metastasise. On the other hand, Type II carcinomas comprise high-grade serous and high-grade endometrioid types with high frequencies of TP53 mutations [17]. High-grade carcinomas are less differentiated with morphology very different from normal tissues. Type II patients respond well to initial platinum-based chemotherapy, unlike many patients with Type I disease [41]. Usually, the prognosis of Type I patients is better than that of Type II patients, as the latter patients are more likely to present with symptoms at advanced stages of the malignancy [97].

A more recent approach to the classification of epithelial ovarian cancer has led to the latest grouping of ovarian carcinomas, whereby the different subtypes are classified under five histological groups, namely high-grade serous ovarian cancer (HGSOC), endometrioid, clear cell, mucinous and low-grade serous ovarian cancer (Table 1.1) [61, 102]. There is an increasing appreciation that these subtypes with different histological, clinical and molecular abnormalities, represent separate disease entities, indicating different treatment approaches [103]. Globally, the most common subtype is HGSOC with over 70% incidence rate, followed by endometrioid and clear cell with 10% incidence rate each, and low-grade serous and mucinous carcinoma, with less than 5% incidence rate each [104].

	HGS	Endometrioid	Clear cell	Mucinous	LGS
Incidence rate (%)	>70	10	10	<5	<5
Putative tissue of origin	Fallopian tube	Endometriosis	Endometriosis	Undefined	Serous borderline tumour
Overall stage at diagnosis	Advanced	Mid-stage	Early	Early	Advanced
Overall prognosis	Poor	Good	Moderate	Moderate	Moderate
Response to 1st line treatment	High	High	Low	Low	Low
Genetic anomalies	*TP53, BRCA1/2, CCNE1, RB1*	*PTEN, CTNNB1, PIK3CA, ARID1A*	*PTEN, PIK3CA, ARID1A*	*KRAS, HER2* amp	*KRAS, BRAF*
Risk Factors	HT	Endometriosis, HT, BMI, age at menopause	Endometriosis, age at menopause, BMI	Smoking, BMI	Endometriosis, BMI
Protective Factors	Parity, lactation, tubal ligation, OCP	Tubal ligation, OCP, lactation	Tubal ligation, parity	Parity, lactation, tubal ligation, OCP	Not established

Table 1.1: Characteristics of epithelial ovarian cancer subtypes

Adapted from [19], [61], [104], and [105]. Where HGS refers to High-Grade Serous, LGS refers to Low-Grade Serous, amp refers to amplification, OCP refers to oral contraceptive pill, BMI refers to body mass index, HT refers to hormone therapy.

1.3.1 High-grade serous ovarian cancer

HGSOC is the most common ovarian carcinoma, which is now thought to frequently arise from the fallopian tube. Patients generally present at late stages of the disease, hence the prognosis is often poor. Fortunately, more than 80% of patients respond well to the primary platinum ± taxane therapy [41]. Less than 20% of patients present with intrinsic resistance. Nevertheless, about 70% of cases relapse 6 months or more after initial treatment [106]. Patients are then given further chemotherapy, to which 30-60% respond, depending on how long it took until relapse [106]. Alas, approximately 80% of the late stage HGSOC patients acquire resistance to initial platinum therapy [107],

with only 10% responding to second line therapy [106]. There are currently limited treatment options for these patients.

A primary hallmark of HGSOC is the presence of TP53 mutations, which occur in almost 100% of HGSOC cases, and are often linked with its genesis [108]. TP53 mutations are also found in the distal fallopian tube, which suggests either a pronounced exposure of this tissue or higher sensitivity of these cells to DNA damage, however, the cause of this DNA damage is unknown [109]. Other genetic anomalies that characterise HGSOC are *BRCA1* and *BRCA2* inactivation, occurring in approximately 5-10% [110-112] and 6% [111, 113] of ovarian cancer patients respectively. Women with germline BRCA mutations have a 30-70% chance of developing ovarian cancer [114]. These mutations can also arise sporadically [61]. A sequence of events proposed by Bowtell relates TP53 mutations and BRCA mutations as follows: an initial *TP53* loss, followed by BRCA loss, leads to homologous recombination repair deficiency, which ultimately causes chromosomal instability and widespread copy number changes [109]. Other important mutations characterising HGSOC include *PTEN* loss, *CCNE1* amplification and *RB1* loss [61].

1.3.2 Endometrioid ovarian cancer

Endometrioid ovarian cancer had traditionally been subdivided into low-grade and high-grade disease. However, as the histology and gene expression of high-grade endometrioid resembles more HGSOC, the term endometrioid nowadays is being restricted to low-grade endometrioid disease, which is thought to originate from endometriosis [105, 115]. Distinguishing factors between endometrioid and high-grade serous include the absence of WT1 [116, 117], and TP53 [118] in ovarian endometrioid cancer cells. Patients presenting with endometrioid carcinoma have good prognosis, as they are often diagnosed at early to mid-stage of the disease, and its response to first line platinum therapy is frequently positive [115]. The most common mutations occur in *CTNNB1* in 30-50% of patients, and these are linked to better outcome, squamous differentiation and low tumour grade [119]. *PTEN* is mutated in about 20% of endometrioid carcinomas, which leads to inhibition of apoptosis [120, 121]. *PIK3CA* gene mutations occur in 20% of these cancers, and these are often

associated with a worse prognosis [122]. Mutations in *ARID1A*, which is known to regulate transcription also occur in these cancers [123].

1.3.3 Clear cell ovarian cancer

Clear cell cancer patients frequently present with advanced stages of the disease, do not respond to platinum therapy as well as HGSOC, and often have poor prognosis [124, 125]. Similar to endometrioid carcinoma, clear cell carcinoma is also associated with endometriosis, and when these patients present during early stages, their prognosis is better [125]. Genetic defects in this subgroup of cancers are also similar to those found in endometrioid carcinoma, with frequent mutations in *ARID1A*, *PIK3CA* and *PTEN* [126, 127].

1.3.4 Mucinous ovarian cancer

Patients with mucinous carcinoma often present at earlier stages than HGSOC, and are not usually sensitive to first line platinum therapy. Mucinous carcinoma is thought to originate from transitional cell nests at the tubal-mesothelial junction, and are frequently borderline tumours [128]. Some mucinous tumours are thought to metastasise from the gastrointestinal tract [105]. Because of their possible gastrointestinal origin, *KRAS* genetic mutations are frequently found in mucinous cancers of intestinal differentiation [129]. They often also present with HER2 amplification [130].

1.3.5 Low-grade serous ovarian cancer

Low-grade serous ovarian cancer patients usually present at early stage and patients are frequently of young age, with the disease following a comparatively indolent course [105]. The progression from benign serous cystadenoma to borderline serous tumour leads to low-grade serous ovarian carcinoma. This disease does not normally progress to HGSOC. Genetic mutations in *BRAF* and *KRAS* characterise low-grade serous carcinoma [131].

1.4 HER signalling pathway involved in ovarian cancer

The human epidermal growth factor receptor (HER) family has been associated with progression in several types of cancer including breast, lung and colon cancers [132]. In ovarian cancer, amplification and/or high expression of epidermal growth factor receptor (EGFR), HER2 and HER3 receptors have been implicated in the progression and prognosis of the disease [133-136].

The HER family (also known as the erythroblastic leukaemia viral oncogene (erbB) family), exist on the cell surface as monomers in the absence of ligand activation. There are four members of this family, EGFR (HER1/erbB1), HER2 (neu/erbB2), HER3 (erbB3) and HER4 (erbB4) (Figure 1.5). The common structure of the HER family of receptors consist of an extracellular domain, a transmembrane domain and an intracellular tyrosine kinase domain with C-terminal domain [137]. With the exception of HER2, ligands bind to their extracellular domain and form homo- or heterodimers with other members of the family, preferentially with HER2 which has the most favourable kinase activity and exists in an activated form [138]. HER ligands are divided into three groups; those which bind specifically to EGFR (epidermal growth factor/EGF, amphiregulin and transforming growth factor-α/TGFα), others conferring dual specificity for EGFR and HER4 (betacellulin, heparin-binding EGF, and epiregulin), and those which bind to HER3 and HER4 (neuregulins/ heregulins (HRG)) [139]. HRG preferentially induces HER2-HER3 heterodimerisations [140], and its overexpression in breast cancer has been associated with poor prognosis [141]. The kinase domain and tyrosine residues which are phosphorylated will be dependent on the type of HER dimerization activated. The phosphorylated residues operate as docking sites which successively activate the downstream signalling response [137].

1.4.1 Downstream signalling pathways

Upon receptor dimerization, multiple downstream pathways are activated which regulate cell proliferation, differentiation, angiogenesis, survival and cellular metabolism amongst other functions. Heterodimerisation allows for a myriad of phosphotyrosine residues to bind, which in turn increases the signalling pathway possibilities [142]. These pathways (Figure 1.5) include, the mitogen activated protein

kinase (MAPK) / ERK pathway that regulates cellular proliferation and invasion; the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway which regulates cell survival and metabolism; the phospholipase Cγ (PLCγ) pathway which controls calcium-dependent actions and the signal transducer and activation of transcription (STAT) pathway which governs cell proliferation and differentiation [138, 143, 144].

In tumourigenesis, mutations within components of these pathways can cause cancer cells to acquire certain aptitudes, including impartiality of proliferation signals, circumvention of apoptosis, insensitivity to growth inhibitory signals, augmented replicative potential and the capability to metastasise [145].

The PI3K/Akt and MAPK pathways regulate migration through a variety of downstream signalling pathways, [146] including, rho-family GTPases, which control cytoskeletal regulators; integrins and associated matrix adhesion proteins; enzymes which degrade ECM proteins, facilitating cell invasion as they reduce adhesive contacts; cell-cell adhesion components; transcription factors which regulate proteins such as matrix metalloproteinases, cadherins and actin that control migration.

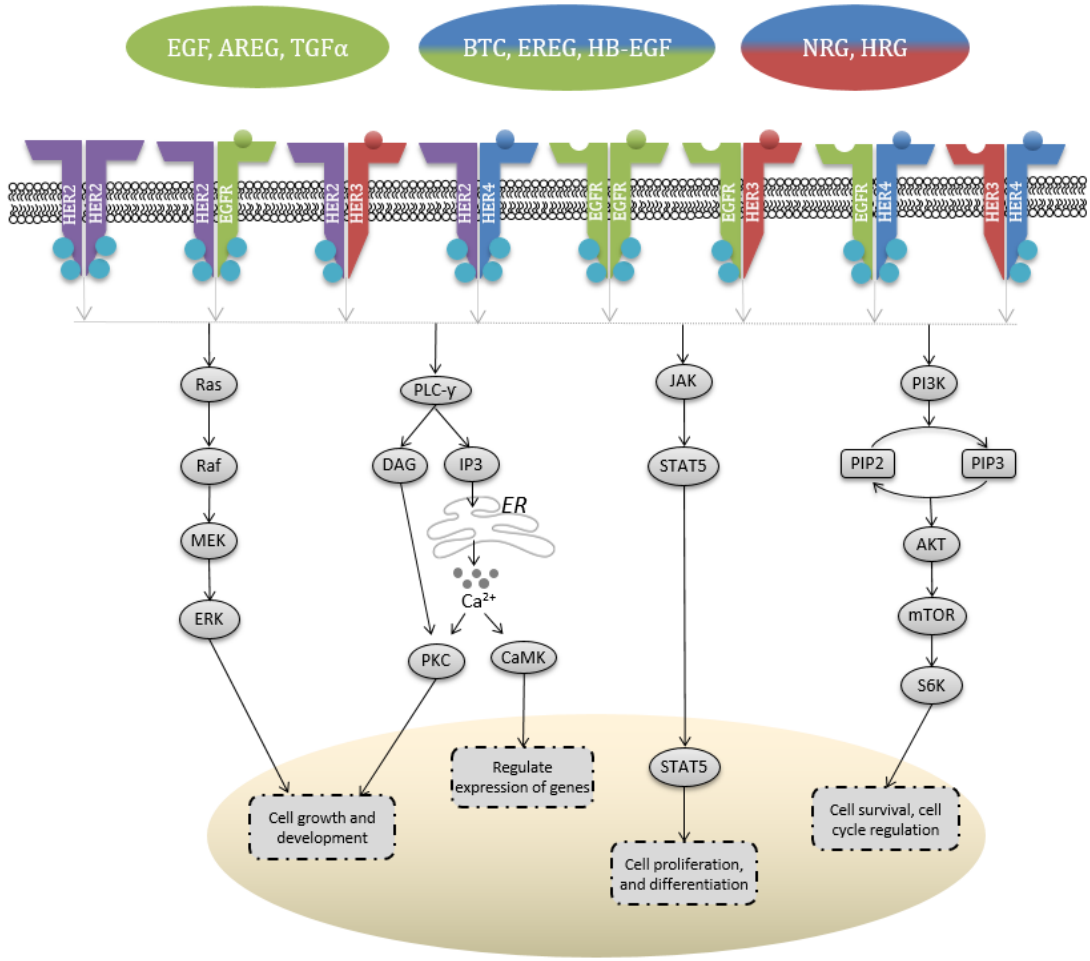


Figure 1.5: Cell proliferation pathways regulated by HER-family members

The downstream pathways regulated by HERs are the MAPK, PI3K/Akt, STAT and PLCγ pathway. Where MEK= Mitogen-activated protein kinase, ERK= extracellular signal-regulated kinases, PLCγ= phospholipase Cγ, DAG= diacylglycerol, IP3= inositol trisphosphate, PKC= protein kinase C, CaMK= calcium-calmodulin dependent kinase, JAK= Janus kinase, STAT= signal transducer and activator of transcription, PI3K= Phosphoinositide 3-kinase, PIP= Phosphatidylinositol (3,4,5)-trisphosphate, Akt= protein kinase B , mTOR= mechanistic target of rapamycin, S6K= Ribosomal protein S6 kinase beta. Adapted from [147].

The **MAPK pathway** is regarded as a paradigm for signal transduction in molecular biology. A signal is propagated along the pathway in response to stimuli via sequential phosphorylation of three kinases, specifically MAPKKK (e.g. B-Raf), MAPKK (e.g. MEK1/2) and MAPK (e.g. ERK1/2) [148]. There are six different groups of MAPKs in mammals, including ERK1/2, ERK3/4, ERK5, ERK7/8, JNK1/2/3 and p38/ERK6 [145]. The location of ERK when it phosphorylates target molecules determines its effects within the cell. In the cytoplasm, it affects cellular movement, metabolism, cell adhesion and nodal regulation of other pathways [149, 150], whereas in the nucleus,

phosphorylated ERK promotes cell cycle progression [151]. Sprouty and Spred, are cytoplasmic proteins which inhibit the MAPK pathway by decreasing phosphorylation [151, 152]. Additionally, ERK can promote tumour suppression through senescence [153] which acts through terminal cell cycle arrest [154].

Mutations in EGFR (eg del19EGFR), *KRAS* and *BRAF* can result in constitutive activation of the MAPK pathway, leading to induction and progression of tumourigenesis. There are some inhibitors acting on these oncogenes, such as vemurafenib, which inhibits BRAF and is currently being used in melanoma [155]. Selumetinib is another inhibitor of the MAPK pathway, which acts on the MEK protein [156]. A phase II clinical trial featuring selumetinib in recurrent low-grade serous ovarian cancer patients resulted in a 15% overall response rate (ORR) and median PFS of 11 months [157]. The response was not correlated with any mutations in *BRAF* or *KRAS* [157]. On the other hand, an investigational drug, CI-1040 was found to be effective *in vitro* in *BRAF* or *KRAS* mutated ovarian cancer cell lines [158].

In the **PI3K/ Akt/ mTOR pathway,** upstream stimulation of the HER receptors results in the eventual phosphorylation of Akt and PDK1 kinases. The binding of PI3K to its ligands causes allosteric activation, leading to the conversion of PIP2 to PIP3. The latter triggers PDK1 proteins and recruitment of Akt proteins in the plasma membrane. Active Akt controls downstream mediators such as mTOR. The latter in turn phosphorylates ribosomal S6 kinase-1, which is essential for translation of mRNA and crucial for cell cycle progression from G1 to S phase [159]. PTEN dephosphorylates PIP3 to PIP2, acting as a negative regulator of this pathway [160]. Recently, phase I and phase II clinical trials of mTOR inhibitors such as temsirolimus and everolimus, have demonstrated positive results in ovarian cancer [161-163]. However, none has yet been approved for the treatment of ovarian cancer, as the outcome to treatment was insufficient.

This pathway is frequently driven by the genetic mutation and amplification of key pathway components, namely loss of *PTEN*, somatic activating mutations in *PIK3CA*, mutations in *PIK3R1*, *EGFR*, *HER2* and *AKT2* in ovarian cancer [148, 164, 165]. Most PI3K/Akt related mutations occur in clear cell ovarian cancer and endometrioid

ovarian cancer [120, 121, 126, 127]. Perifosine, an Akt inhibitor demonstrated synergistic inhibition when combined with paclitaxel in ovarian cancer patients [166].

The **PLCγ pathway** is associated with calcium dependent cellular functions such as cell proliferation, migration, inflammation and gene expression. PLC-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate produces the messenger molecules inositol 1,4,5-trisphosphate (increases the intracellular free Ca2+) and diacylglycerol (activates protein kinase C), mediated by the PLC-γ isozymes [167]. Phospholipases transmit their signals via lipid mediators to produce calcium in the endoplasmic reticulum [168]. An *in vitro* ovarian cancer study linked PLCγ to migratory ability of the cells [169].

The **STAT pathway** consists of seven cytoplasmic transcription factors, which are activated by receptor tyrosine kinases to form dimers, which migrate to the nucleus to control gene expression of proteins whose up-regulation leads to uncontrolled cell proliferation and cell cycle progression [170]. The STATs are not only involved in carcinogenesis and progression, but also contribute to cancer cell survival. Inhibiting these STATs causes apoptosis of tumour cells [171]. Different STATs play various functions. This pathway is often linked with several cancer types, namely ovarian cancer, whereby STAT3 and STAT5 play an important role [172], and are involved in cell cycle progression, angiogenesis and inhibition of apoptosis. STAT proteins can be directly activated by HER through one of their domains, SH2 [173]. However, STAT activation could also occur indirectly by the activation of inflammatory cytokines, such as IL6 [174].

1.4.2 HER receptor significance in ovarian cancer

1.4.2.1 EGFR

The EGFR (HER1) is a protein-tyrosine kinase receptor comprising an extracellular ligand-binding domain with 622 residues, a 23-residue transmembrane segment, and a large, 522-residue intracellular domain [142]. EGFR plays an important role in epithelial cell development, as this process is often regulated by EGF [175]. EGFR has multiple tyrosine phosphorylation sites [176], which determine the downstream

signalling cascade initiated by EGFR activation and these include Tyr1068, Tyr1148, Tyr1173, Tyr992 and Tyr1086 [177].

EGFR is normally only weakly expressed in the ovary, however multiple studies have demonstrated that EGFR can be highly expressed in ovarian cancer. Immunohistochemistry studies have indicated that 30-70% of ovarian cancers have EGFR expression [178-180]. High expression of EGFR is associated with poor PFS, advanced tumour grade, greater residual tumour mass and rapid proliferation [133, 134, 136]. It has also been suggested that high EGFR expression in tumour stroma is associated with aggressive clinical conditions, and EGFR up-regulation in fibroblasts is associated with growth and migratory abilities of ovarian cancer cells [181]. Several studies have reported EGFR expression as being an independent prognostic factor of poor outcome in ovarian cancer [182, 183]. Additionally, recent studies indicate that activation of EGFR during platinum treatment leads to the development of platinum resistance [184, 185].

1.4.2.2 HER2

The HER2 receptor consists of 1255 amino acids and is a 185 kD transmembrane glycoprotein [186]. It lacks a ligand-binding domain, thus it requires other HERs for dimerization. However, when HER2 is overexpressed, it is able to form homodimers which are able to elicit downstream signalling responses [187]. HER2 is thought to have the strongest catalytic kinase activity, thus HER2-containing heterodimers have the greatest signalling capacity, and HER2-HER3 heterodimers are considered to be the most potent when it comes to signalling [188]. HER2 has multiple phosphorylation residues including Tyr1139, Tyr1196, Tyr1221/1222 and Tyr1248 [189].

HER2 is overexpressed in approximately 6-30% of ovarian cancer patients [134, 190] and was initially associated by Slamon *et al.* with DNA amplification and poor prognosis [134, 191, 192]. Overexpression is often found in the mucinous (19%) [193] and clear cell (14%) [194] subtypes. A study by Mccaughan *et al.* found that some serous (3%) and endometrioid ovarian cancers (2%) have HER2 overexpression also [195]. These data were reanalysed recently to reflect the new ovarian cancer classification, and it was found that 8.5% of endometrioid and 5.1% of HGSOC were

HER2 amplified (personal communication- S. Langdon, 2018), suggesting significant percentages of these more frequent subtypes possess HER2 amplification.

1.4.2.3 HER3

HER3 is expressed as a full-length receptor on the cell surface at a size of 180 kD, in parallel with truncated intracellular iso-forms. However, the activity of the latter is not well defined [196, 197]. HER3 has very weak protein tyrosine kinase activity, hence transphosphorylation by other HER members would result in stronger propagation of cell signals [142, 198].

HER3 is more frequently expressed in ovarian cancer (30-80%) than EGFR and HER2 [199] and is more common amongst borderline and early-stage lesions [200]. The most potent signalling complex among the dimerization possibilities is generated when HER3 heterodimerises with HER2 [201]. High HER3 expression has been associated with poor clinical outcome; average survival time for patients with low HER3 expression was 3.3 years, in contrast to 1.8 years for patients with high HER3 expression [135]. Studies in various cancer types show that when HER3 and MET are both highly activated, they are often associated with therapy resistance [202-204]. Other studies show that high expression of HER3 may lead to a HER3-PI3K-Akt signalling cascade in doxorubicin and cisplatin treated ovarian cancer, which often leads to therapy resistance [205, 206].

1.4.2.4 HER4

HER4 is the least understood receptor of the HER family. It occurs in spliced isoforms, often being further processed by enzymes into a soluble intracellular domain which can disperse to the cell cytoplasm or nucleus [207]. In breast cancer, the nuclear localization of the intracellular domain in combination with oestrogen expression, predicted for worse clinical outcomes compared to membrane HER4 and oestrogen [208].

There are conflicting views about the presence of HER4 in ovarian cancer, with earlier reports finding decreased or absent presence of the receptor [209], while more recent studies suggests an increased presence of HER4 in malignant tissue than normal tissue

[199, 210, 211]. Although the implication of HER4 expression in ovarian cancer is unclear, two studies found a possible correlation between HER4 expression and serous ovarian cancer resistance to chemotherapy [210, 212].

1.5 Monoclonal antibodies

Conventional chemotherapy often does not discriminate normal cells from tumour cells, therefore can lead to multiple side effects. Conversely, targeted therapy is designed to affect molecular targets which have a role in irregularly dividing cells. Often, they are used in combination with radiotherapy or chemotherapy for an additional or synergistic effect [213].

Monoclonal antibodies (mAbs) are immunoglobulins that recognise specific binding sites or epitopes on an antigen [214]. They consist of constant heavy chains and light chains, with the latter having a variable region, which is the antigen binding/recognition region. To overcome immunogenicity, "humanised" chimeric mouse-human antibodies have been developed [215]. HER-targeted mAbs are currently being used widely in the treatment of breast cancer [216, 217], and colorectal cancer [218, 219]. Recent *in vitro* and *in vivo* preclinical studies and clinical trials have been focusing on the role of mAbs targeted against HER2 in ovarian cancer, especially given that selected subtypes, particularly mucinous cancers, have HER2 amplification and overexpression [130, 220]. However, to date there are no HER-targeted mAbs approved for the treatment of ovarian cancer.

1.5.1 Trastuzumab

The first HER2-targeted mAb to be approved for use in oncology was Trastuzumab (Herceptin®) in 1998. Trastuzumab is indicated in metastatic breast cancer overexpressing HER2 as monotherapy or in combination with paclitaxel depending on whether the patient had received chemotherapy regimens for their metastatic disease [221]. It is a recombinant humanized mAb, which binds to the juxtamembrane region of the extracellular HER2 domain, inhibiting extracellular domain cleavage, obstructing HER2 homodimerisation as well as inducing antibody dependent cell-

mediated cytotoxicity [222, 223] (Figure 1.6). In breast cancer, trastuzumab requires HER2 overexpression for its effects [223, 224].

A phase II trastuzumab clinical trial involving 41 eligible ovarian cancer patients with HER2 overexpression, demonstrated an ORR of 7.3%, which included one complete and two partial responses. The median PFS was two months [225].

1.5.2 Pertuzumab

Pertuzumab (Perjeta®) was approved in 2012 for the treatment of HER2 positive metastatic breast cancer patients who had not been previously exposed to anti-HER2 therapy or chemotherapy for metastatic disease and for the neoadjuvant treatment of HER2 positive early stage breast cancer [226]. Pertuzumab acts by blocking the dimerization domain on HER2, thus inhibiting HER2 hetero-dimerization [223] (Figure 1.6). Unlike trastuzumab, it still produces inhibitory effects in the absence of HER2 overexpression [227, 228].

In a phase III breast cancer clinical trial (CLEOPATRA), the combination of trastuzumab and pertuzumab plus docletaxel showed additional benefit compared to monotherapy, which subsequently led to the approval of the combination for HER2 positive metastatic breast cancer [229]. Pertuzumab was assessed in a phase III clinical trial (PENELOPE) in platinum-resistant ovarian cancer featuring low HER3 expression, after a randomised phase II clinical trial with pertuzumab showed a better PFS (5.3 months vs 1.4 months with placebo) in patients with low HER3 expressing, platinum-resistant ovarian cancers [230, 231]. In the PENELOPE trial, the addition of pertuzumab to chemotherapy produced a PFS of 4.3 months and an ORR of 13.1%, when compared to the PFS of 2.6 months and ORR of 8.7% in placebo plus chemotherapy patients [231]. In the *in vivo* setting using ovarian cancer xenografts, our group has demonstrated that the combination of pertuzumab and trastuzumab produces more prolonged growth inhibition, when compared to either antibody used as single agent [232]. Furthermore, another preclinical study has also suggested that trastuzumab could augment sensitivity in ERα-positive ovarian cancer to endocrine therapy [233].

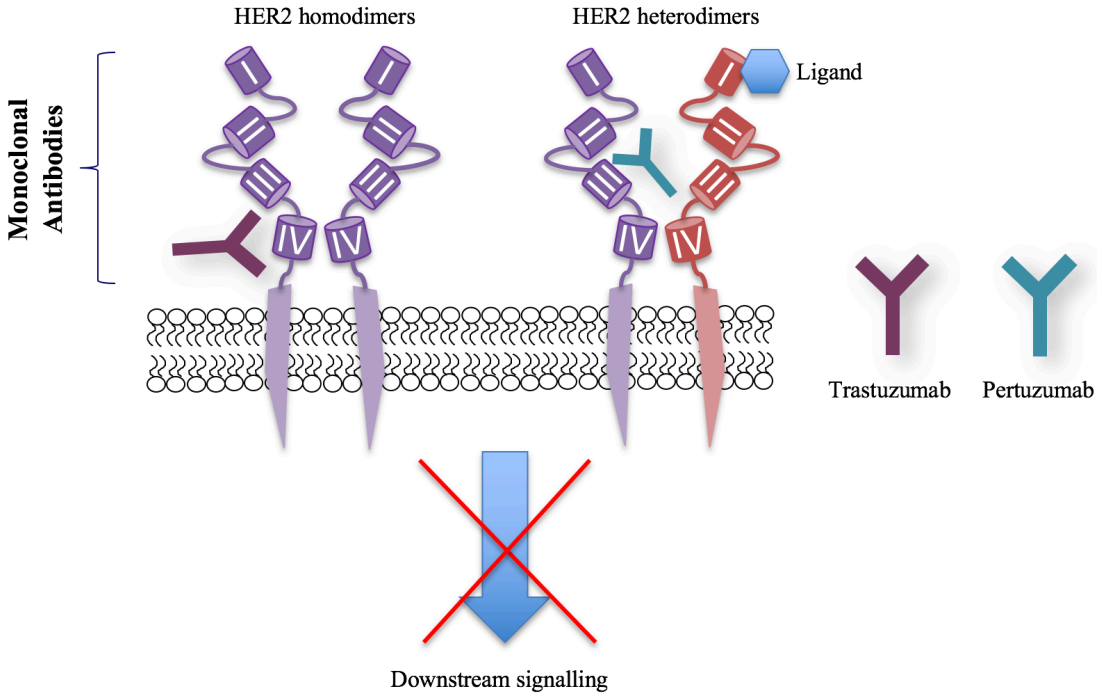


Figure 1.6: Mode of action of HER2-targeted mAbs

The HER-targeted mAbs act on homo or heterodimers that involve HER2 to prevent the downstream signalling pathways brought about by the HERs.

1.5.3 Trastuzumab-emtansine

Trastuzumab-emtansine, T-DM1 (Kadcyla®) is a HER2 targeted mAb conjugated to a microtubule inhibitor (emtansine). It was approved in 2013 for the treatment of recurrent advanced or metastatic breast cancer [234]. T-DM1 acts by binding to HER2, triggering the endocytosis of the HER2-T-DM1 complex. Once in the cytoplasm, DM1 is released from the complex and inhibits microtubule assembly, leading to cell death [235]. In a phase III breast cancer clinical trial, the T-DM1 treated cohort showed a superior clinical outcome (PFS 9.6 months) compared to patients who received lapatinib plus capecitabine (6.4 months) [236]. It was later approved as monotherapy for HER2 positive metastatic breast cancer resistant to trastuzumab treatment [237]. In a recent *in vivo* study by Menderes *et al*., T-DM1 demonstrated superior tumour growth inhibitory activity against HER2 overexpressing ovarian cancer primary cell lines, than pertuzumab, trastuzumab and their combination [238]. It also improved survival in a xenograft model [238]. This agent was also shown to have similar antibody-dependent cell-mediated cytotoxicity as pertuzumab, trastuzumab and their combination [238]. Another study demonstrated excellent activity against both

subcutaneous and intraperitoneal growth of the SKOV3 ovarian cancer xenograft model [239].

1.5.4 Other HER-targeted antibodies

Cetuximab (Erbitux®) is an EGFR-targeted mAb that is currently being used in the treatment of colorectal cancer [218]. It is active in patients who do not harbour a *KRAS* mutation and who possess *EGFR* amplification [240, 241]. In a phase II trial using cetuximab plus carboplatin in recurrent platinum-sensitive ovarian cancer patients, there was a response rate of almost 27% in EGFR-positive patients [242]. This contrasts to a 9% response rate in EGFR-positive patients when cetuximab was used as monotherapy [243].

Panitumumab (Vectibix®) is also an EGFR-targeted mAb which has been approved in 2006 for the treatment of recurrent metastatic colorectal carcinoma [219]. Panitumumab requires a WT *BRAF* and *KRAS* phenotype for optimal therapeutic effect [244, 245]. Panitumumab with pegylated liposomal doxorubicin was effective in a phase II platinum-resistant ovarian cancer study, with an OS of 8.1 months [246]. A phase II clinical trial investigating the administration of panitumumab with platinum-based chemotherapy in relapsed platinum-sensitive patients, revealed that this combination did not offer advantages over the standard treatment [247].

Seribantumab is an anti-HER3 mAb which was previously in phase II clinical trials [248]. Interestingly, from a phase II clinical trial on platinum-resistant or refractory ovarian cancer patients, it emerged that detectible levels of HRG and low levels of HER2 were important for its therapeutic effects [249].

1.6 Tyrosine kinase inhibitors

Tyrosine kinases are enzymes that catalyse the phosphate transfer from adenosine triphosphate (ATP) onto a tyrosine residue within a target protein. This transfer may then elicit a further response within a signalling pathway. Cellular molecules that possess tyrosine kinase activity include the HER family, VEGF receptors, platelet-

derived growth factor receptors (PDGFR), and also non-receptor tyrosine kinases BCR-ABL and KIT among others [213, 250].

The tyrosine kinase inhibitors (TKIs) are small molecular drugs which inhibit tyrosine kinases, and can be classified into three main types. Most small molecule TKIs are type I, which compete with ATP by binding to ATP binding sites within the active conformation of the receptors, thereby interfering with the phosphorylation of tyrosine residues and inhibiting activation of downstream pathways [250, 251]. Type II TKIs bind to the inactive conformation of the kinase, while type III allosteric inhibitors bind to sites distant from the active site [252]. First generation TKIs included the EGFR inhibitors, gefitinib and erlotinib, which have shown clinical efficacy against EGFR mutant lung cancer [253, 254]. Since a resistance mutation at T790M frequently develops on treatment [255], covalent irreversible 2nd generation TKIs were developed [256]. These include afatinib and neratinib, which are active against this mutation. Other HER inhibitors were developed with broader acting inhibitory activity across multiple HER family members (pan-inhibitors) and these include lapatinib and canertinib as early developed inhibitors, followed by neratinib, sapitinib and dacomitinib. Finally, multitargeted TKIs that target not only the HER family but also other targets (e.g. PDGFR, VEGFR etc) include vandetininib and leflunomide.

The HER-targeted TKIs differ in their side effect profiles, however they are generally well tolerated. The main side effects include skin/ hair toxicity, facial hair growth and erythema, haematological side effects (eg anemia, thrombopenia), oedema, gastric effects and hypothyroidism. Cardiac toxicity is one of the major long term adverse effects [257].

1.6.1 First generation TKIs

1.6.1.1 Erlotinib

Erlotinib (Tarceva®; OSI-774) is a quinazoline derivative, reversibly inhibiting EGFR (Table 1.2). It is indicated as first line therapy in non-small cell lung cancer (NSCLC) and in combination with gemcitabine in pancreatic cancer [258]. Erlotinib has shown enhancement of PFS when compared to chemotherapy (13.1 months vs 4.6 months

respectively) in chemotherapy-naïve NSCLC patients, harbouring *EGFR* mutations [259]. An *in vivo* study in EGFR high expressing ovarian cancer demonstrated that a combination of erlotinib plus olaparib, a PARP inhibitor, had a greater tumour suppressor effect than the monotherapy alone [260]. A phase II study combining erlotinib with carboplatin showed that the combination was more effective in ovarian cancer patients with platinum-sensitive disease rather than platinum-resistant disease, with 57% and 7% objective response rates respectively [261]. This finding could be attributed predominantly to the anti-tumour activity of carboplatin. An exploratory phase II clinical trial involving bevacizumab and erlotinib determined that high levels of VEGF-A caused bevacizumab resistance, while erlotinib did not seem to contribute to the efficacy of the combination [262]. A randomised phase III study evaluated the efficacy of administering erlotinib in patients after first line chemotherapy for ovarian cancer. The median PFS in patients receiving erlotinib and placebo was 12.7 and 12.4 months respectively, concluding that erlotinib does not provide pre-treated ovarian cancer patients with additional benefits [263]. In an extension to this phase III clinical trial, a tissue biomarker study concluded that increased EGFR gene copy number led to worse OS and PFS [264].

1.6.1.2 Gefitinib

Gefitinib (Iressa®, ZD-1839) is a type I quinazoline derivative TKI, reversibly selective for EGFR (Table 1.2). Gefitinib is marketed for monotherapy of locally advanced or metastatic NSCLC and is most effective in cancers with mutation or increased EGFR copy number [265]. Preclinical studies demonstrated that combining gefitinib with cisplatin, increased the efficacy of cisplatin, mainly due to EGFR downstream inhibition and blockade of DNA repair mechanisms of ovarian cancer [266]. When evaluated in an ovarian cancer phase II trial, gefitinib showed a limited response rate in platinum pre-treated ovarian cancer patients, with only one of twenty-seven patients indicating an ORR of 4% [267]. However, this patient was the one participant in this cohort harbouring a mutation in the catalytic region of the EGFR, consistent with data for NSCLC. Another phase II clinical trial combining gefitinib with tamoxifen in refractory or resistant ovarian cancer patients, did not demonstrate

any advantageous tumour responses with median time-to-progression being 58 days [268].

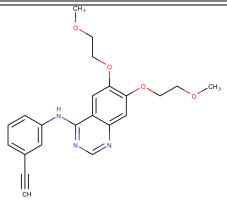
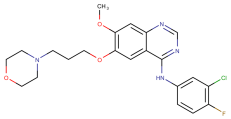
TKIs	Structure	Pharmacology	Clinical status	IC_{50}: HERs (nM)	Average $[Plasma]_{max}$ (µM)
Erlotinib (OSI-774)		Specific and reversible inhibitor of EGFR	Marketed for NCSLC and pancreatic cancer	EGFR: 20	4.5 [269]
Gefitinib (ZD-1839)		Specific and reversible inhibitor of EGFR	Marketed for NCSLC	EGFR: 14	0.3 [270]

Table 1.2: Main characteristics of first generation HER-targeted TKIs

1.6.2 Second generation TKIs

1.6.2.1 Afatinib

Afatinib (Gilotrif®, Giotrif®; BIBW-2992) is a type I anilinoquinazoline derivative TKI inhibitor, which irreversibly binds to EGFR, HER2 and HER4 [271] (Table 1.3, Figure 1.7). It is currently approved for the treatment of *EGFR*-mutated NSCLC. *In vitro* studies using ovarian cancer cell line models showed that afatinib possessed anti-migration and anti-proliferation properties [272]. In an ovarian cancer preclinical study, afatinib reversed ABC-mediated multidrug resistance to paclitaxel and adriamycin, as well as increased the apoptosis efficacy of paclitaxel in ABCB1 overexpressing tumours [273]. *In vivo*, afatinib plus docetaxel showed enhanced tumour size reduction, than either drug as a single agent [274]. It was also found effective in inhibiting basal and HRG-induced EGFR, HER2, Akt and ERK phosphorylation [272]. Afatinib has not been extensively tested in ovarian cancer in clinical trials. However, a phase I dose escalation study which included four ovarian cancer patients showed clinical promise as indicated by stable disease [275, 276]. In breast cancer, afatinib treatment was effective as it overcomes HER^{T798I}-mediated neratinib resistance [277], however it produced side effects in patients [278, 279].

1.6.2.2 Canertinib

Canertinib (CI-1033; PD-183805) is a 4-anilinoquinazoline, irreversible, pan-HER TKI, which reached phase II clinical trials, however it was recently withdrawn [280] (Table 1.3, Figure 1.7). *In vitro*, it enhances the intracellular accumulation of certain cytotoxic drugs, in cells overexpressing ATP-binding cassette subfamily G2 (ABCG2) transporter [281]. Canertinib showed potent inhibitory effects in ovarian cancer cell lines, especially when combined with a c-MET inhibitor (PHA665752), which further reduced phosphorylation and total expression of signalling pathway [272, 282, 283]. A randomised phase II clinical trial in platinum-resistant or refractory ovarian cancer patients, resulted in disease stability in about 30% of patients, with median PFS and OS of 2.2 months and 9.1 months respectively [284]. Studies suggest that high HER levels and low HER autocrine ligands lead to canertinib resistance [285].

1.6.2.3 Lapatinib

Lapatinib (Tyverb®; GW-572016) is an oral competitive TKI inhibitor selective for EGFR and HER2 (Table 1.3, Figure 1.7). It is indicated in HER2 overexpressing breast cancer. Preclinical data indicate that lapatinib works better when HER2 is overexpressed and most likely homodimerised [286]. In breast cancer clinical trials, lapatinib has shown better inhibition when HER2 is expressed rather than when there was EGFR expression [287]. In a phase I study assessing lapatinib in combination with carboplatin in ovarian cancer, whilst there were some non-dose limiting toxicities, half of the patients had partial response or stable disease [288]. The combination of lapatinib and topotecan was assessed in a one-arm phase II trial (LapTop), whereby 20% of patients experienced benefit, however hematologic adverse effects were considerable [289, 290]. In another one-arm phase II study in recurrent ovarian cancer, the median PFS was 1.8 months, OS was 10.5 months, and 8% of patients had PFS at 6 months. The low PFS could be due to low EGFR and HER2 expression [291].

1.6.2.4 Neratinib

Neratinib (Nerlynx®; HKI-272) is an oral irreversible pan-HER inhibitor (Table 1.3, Figure 1.7). It has been recently approved for the adjuvant treatment of early stage HER2-positive breast cancer [292], after a phase III study concluded a 2-year invasive disease free survival rate of 94% when neratinib was given after chemotherapy and

trastuzumab adjuvant therapy versus 92% in the placebo arm [293]. Neratinib has demonstrated pre-clinical efficacy in ovarian cancer, especially in HER2-amplified carcinosarcoma, whereby it inhibits proliferation and tumour growth [294], as well as decreases phosphorylation of transcription factor S6, and causes cell cycle arrest in the G0/G1 phase [295]. There is currently a phase II clinical trial underway assessing neratinib efficacy in HER2-positive solid tumours (SUMMIT Trial) with mutations in EGFR, HER2 or HER3, including ovarian cancer (NCT01953926). This included four evaluable ovarian cancer patients of whom one had stable disease while three had disease progression [296]. In a phase II study assessing neratinib in advanced NSCLC, patients with T790M EGFR mutation did not respond to therapy, however partial responses or disease stabilisation were seen in G719X EGFR mutated patients [297]. In HER2-positive breast cancer, neratinib as a single agent was well tolerated and has shown substantial clinical activity in trastuzumab-naïve patients, with a 16-week PFS rate of 78%, and medial PFS of 39.6 weeks versus 16-week PFS rate of 59% and median PFS of 22.3 weeks in trastuzumab pre-treated patients [298]. The activity of neratinib was correlated with HER2 levels and not with EGFR levels *in vitro* [299]. Other clinical trials in breast cancer assessed neratinib in combination with temsirolimus, vinorelbine, paclitaxel ± trastuzumab and capecitabine, all of which were well tolerated by patients and had anti-tumour properties [300-304].

1.6.2.5 Sapitinib

Sapitinib (AZD 8931) is a type I, reversible, equipotent inhibitor of EGFR, HER2 and HER3 receptor signalling, especially when HER2 is not overexpressed while EGFR is highly expressed [286, 305] (Table 1.3, Figure 1.7). In fact, sapitinib has shown superior tumour growth inhibition against EGFR-driven xenograft tumours when compared to lapatinib [286]. Preclinical studies suggest that sapitinib favours HRG-induced HER2/HER3 heterodimers [286]. It has also demonstrated its ability to inhibit proliferation through the p-ERK and p-Akt pathways, and induces apoptosis through M30 and cleaved caspase-3 [286]. *In vitro* studies suggest that sapitinib in combination with trastuzumab may be an effective therapy in HER2 breast cancer, and it showed efficacy in trastuzumab sensitive and resistant cells [306]. In xenograft inflammatory breast cancer models, sapitinib alone significantly inhibited tumour growth, however

the combination of paclitaxel and sapitinib was more effective than either agent alone [305]. Sapitinib has been tested in eight ovarian cancer patients amongst others in a phase I clinical trial, to assess the maximum tolerated dose, established to be 240 mg twice daily [307].

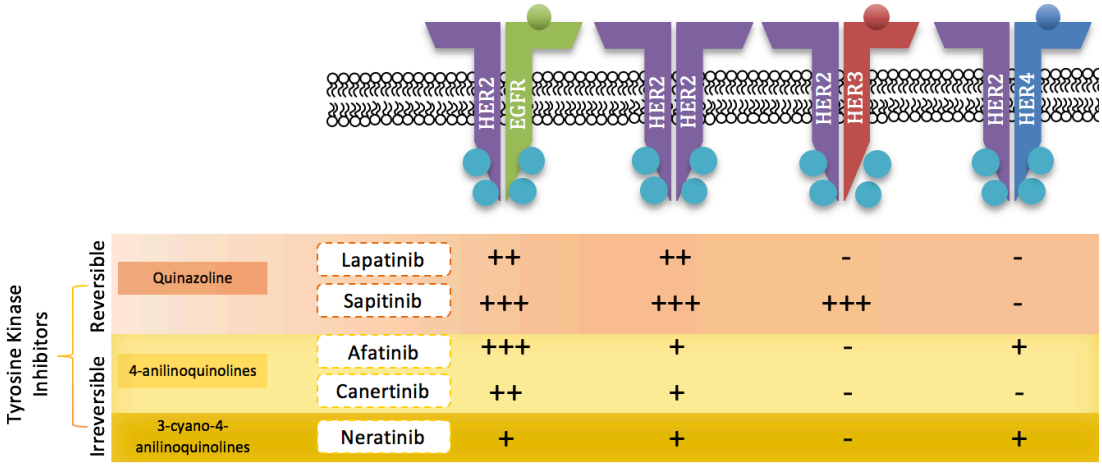


Figure 1.7: Main sites of action of second generation TKIs used in this study

The relative potency is indicated as follows: +++ is very potent, ++ is potent, + is mildly potent and – is generally inactive. Adapted from [147].

TKIs	Structure	Pharmacology	Clinical status	IC_{50}: HERs (nM)	Average $[Plasma]_{max}$ (μM)
Afatinib (BIBW-2992)		Potent and irreversible inhibitor of EGFR/HER2 including erlotinib-resistant EGFR T790 M [308], [309]	Marketed for EGFR mutation positive lung cancer	EGFR: ~3.6 HER2: 14.0	0.06-0.1 [308, 310]
Canertinib (CI-1033)		Irreversible non-selective EGFR family inhibitor, with an additional benefit of blocking mutant EGFRvIII [311]	Discontinued by Pfizer [280].	EGFR: 1.8 HER2: 11.0 HER4: 27.0	0.7-1.5 [312]
Lapatinib (GW-572016)		Reversible and specific inhibitor to EGFR and HER2 [251]	Marketed for HER2 overexpressing breast cancer	EGFR: 10.8 HER2: 9.2 HER3: 13.0 HER4: 367	0.3-2.0 [313]
Neratinib (HKI-272)		Potent irreversible, pan-HER (ie, HER 1, 2, and 4) TKI, with low molecular weight [298]	Marketed for HER2+ breast cancer	EGFR: 92 HER2: 59	0.08-0.12 [314]
Sapitinib (AZD-8931)		Equipotent inhibitor of EGFR, HER2 and HER3 [286].	Phase II clinical trials	EGFR: 4.0 HER2: 3.0 HER3: 4.0	0.5-1.1 [307]

Table 1.3: Main characteristics of second generation of HER-targeted TKIs used in this study.

Adapted from [147, 315].

1.6.3 Biomarkers of response to TKIs in ovarian cancer

In breast and lung cancers, informative biomarkers of sensitivity to HER TKIs include overexpression of HER2 and mutation of EGFR. HER2 overexpression is an effective biomarker of sensitivity to HER2-targeted TKIs such as lapatinib in breast cancer. Preclinical studies of T-DM1 in ovarian cancer suggest that even low HER2 expression was important for the anti-tumour properties of T-DM1 in model systems [316]. Analysis of a series of ovarian cancer xenograft models demonstrated that curative activity with the trastuzumab/pertuzumab combination was obtained in cancers with amplification and overexpression of HER2. There is currently little information to indicate how expression levels of HER2 relate to TKIs in ovarian cancer [232, 233] and there is an interesting case report of a dramatic remission of a chemotherapy-resistant ovarian cancer to trastuzumab which was HER2-negative, suggesting that the rules governing response in ovarian cancer may differ from those for breast cancer [317]. In a phase II platinum-resistant/refractory ovarian cancer trial, an anti-HER3 monoclonal antibody, seribantumab in combination with paclitaxel, was effective in patients with detectable HRG and low HER2 expression [249]. For EGFR, the mutated form with deletions in exon 19 indicates sensitivity to TKIs such as erlotinib and gefitinib in NSCLC [318]. However, the importance of EGFR mutations in ovarian cancer is still not well researched since the incidence is much lower. As mentioned above, in a phase II trial of gefitinib in 27 ovarian cancer patients, the one responding cancer did contain an EGFR mutation (2235del15; E746-A750del) in the catalytic domain consistent with this molecular feature being an indicator of sensitivity [267]. This requires validation in future studies.

1.6.4 Resistance to TKIs

Tumours may become resistant to TKI therapy through various mechanisms. One of them is through EGFR mutation, whereby a mutation at position 790, enhances affinity for ATP rather than TKIs at the ATP binding pocket [319]. Other mutations in EGFR that are frequent include L747S, D761Y, T854A [320]. HER2 overexpression and mutations such as $HER2^{L869R}$ [277, 321] could also lead to TKI therapy resistance. Abnormal activity in pathways also link with resistance, such as the amplification of the c-Met transmembrane receptor tyrosine kinase, overexpression of human growth

factor (ligand for c-Met), overexpression of VEGF receptor (which leads to invasion) and *EGFRvIII* mutation (activates PI3K/AKT/mTOR pathway and increases proliferation), K-RAS mutation (leads to uncontrolled proliferation) and loss of PTEN (which usually inhibits cell migration) [322-325]. Alternative pathways which can bypass control include aberrant activation of MET and HGF [326, 327], modifications in VEGF receptors which trigger vascular permeability, in PDGF which regulate angiogenesis and in interleukin-6 which controls inflammatory processes [328-330]. ATP binding cassette transporters overexpression is also a worrying mechanism of resistance as it reduces drug uptake while increasing drug efflux, resulting in a low drug concentration in the cytoplasm [322].

1.7 Ovarian cell line models

Human ovarian cancer cell lines have provided valuable model systems to study a variety of ovarian cancer characteristics, including chemo-sensitivity, cellular biology and molecular response [41]. A large number of ovarian cancer cell lines have been established and many of these are in widespread use [331, 332]. These models can reflect the diverse characteristics found in clinical specimens, such as varying response to chemotherapy.

A panel of three pairs of human HGSOC cell lines was used in this study. Each pair of cell lines (PEA1 – PEA2; PEO1 – PEO4; PEO14 – PEO23) were established in Edinburgh from the ascites of the same patients at points before and after clinical resistance had developed within the same patient [333] and are described in Table 1.4 and Figure 1.8. These cell lines are an excellent model for investigating response of drugs in naturally-occurring platinum-sensitive and acquired platinum-resistant ovarian cancer. The PEA2 cell line has been shown to express low levels of tumour necrosis factor receptor-associated protein 1 (TRAP1). TRAP1 is a key regulatory in cell metabolism, and cisplatin resistance was linked with increased oxidative metabolism [334]. Recent studies have demonstrated that PEO1 cell line is BRCA2 mutant, whilst its counterpart, PEO4 cell line has a restored BRCA2 status through a secondary mutation [335]. On the other hand, the PEO14-PEO23 cell line pair does not demonstrate any HR or mismatch repair deficiencies but PEO23 acquired a tandem

duplicator phenotype [336]. In general, the platinum-resistant counterpart of these three pairs of cell lines did not experience lower accumulation of platinum agent [337].

Cell line	Patient	Treatment
PEA1	A	No previous treatment
PEA2	A	Relapse from cisplatin and prednimustine treatment; established after 6 months
PEO1	B	Previously received cisplatin, 5-fluorouracil and chlorambucil treatment. Relapsed after 22 months
PEO4	B	Clinical resistance to cisplatin, 5-fluorouracil and chlorambucil treatment; established after 10 months
PEO14	C	No previous treatment
PEO23	C	Relapse from cisplatin and chlorambucil treatment; established after 10 months

Table 1.4: Summary of the characteristics of the HGSOC cell lines used in this study
All cell lines were of HGSOC origin, with mutations in TP53 [331].

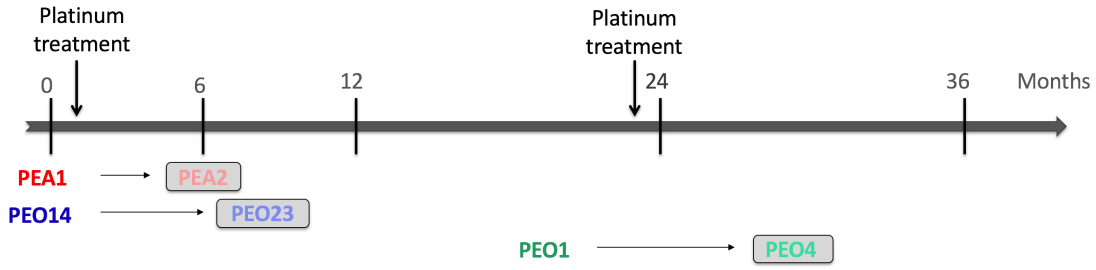


Figure 1.8: An overview of the cell line pairs used in this study
The grey boxes represent the platinum-resistant cell lines [97].

Additionally, the non-HGSOC SKOV3 ovarian cancer cell line was used, which is characterised by its HER2 overexpression [338]. It was established from the ascites of a 64-year old Caucasian female prior to thiotepa treatment [339].

1.8 Aims and objectives

Recent studies in ovarian cancer show that resistance to platinum therapy is often associated with EGFR activation [184, 185]. Additionally, a preclinical study by Sims *et al.*, demonstrates that ovarian cancer xenografts which are normally resistant to platinum therapy were sensitive to mAbs, especially in high HER2 ovarian cancers [232]. A phase II clinical trial of the anti-HER2 antibody pertuzumab suggested that platinum-resistant ovarian cancer might be more sensitive to pertuzumab than platinum-sensitive ovarian cancer [340]. HER2 targeted therapy has demonstrated clear value in the treatment of HER2 positive breast cancer [341]. This approach has not been studied extensively in ovarian cancer, and the possibility of using HER-targeted therapy in platinum-resistant ovarian cancer has only had limited testing.

The hypothesis being explored within this project is whether broad spectrum inhibition of the HER pathway, which is often associated with DNA repair [342, 343], would inhibit key functionality of platinum-resistant HGSOC cells, and whether this sensitivity inhibition could be correlated with the level of expression of HER family members. These findings will be useful as they give an indication whether HER expression could be a possible biomarker to treatment response and if there are plausible alternative therapies to patients presenting with platinum-resistant disease. Broad-spectrum TKIs were investigated in this project as previous studies suggest that more specific TKIs, such as gefitinib and erlotinib, are not as effective in ovarian cancer patients pre-treated with platinum-based chemotherapy [261, 267].

The different subtypes of ovarian cancer are recognised to be different diseases, and merit different treatment approaches. HGSOC is the most common ovarian cancer type, affecting over 70% of epithelial ovarian cancer patients. Therefore, this project aims to evaluate the effectiveness of broad-spectrum HER-targeted TKIs in the treatment of HGSOC, particularly in platinum-resistant cases. This was done with the following objectives:

1. To investigate the proliferative, migratory and invasive response of HGSOC platinum-resistant versus platinum-sensitive ovarian cancer cell lines to HER-targeted mAb and TKI treatment.

2. To evaluate the effect of the combination of TKIs with mAbs or cytotoxics on proliferation.

3. To assess the signalling pathways through which TKIs work when activated by EGF or HRG.

4. To investigate the role of EGFR, HER2 and HER3 on the functional effects of neratinib and other TKIs on cellular functionality by knockdown and transfection studies.

5. To identify expression changes linked to treatment by undertaking gene expression analysis of cell lines in the absence and presence of TKI.

6. To identify potential biomarkers which are HER-targeted therapy-induced and validate these markers in tissue microarrays (TMAs) containing untreated and pertuzumab ± trastuzumab treated tumour cores over a period of time.

2 Materials and methods

2.1 Cell culture

The cell line panel - PEA1, PEA2, PEO1, PEO4, PEO14, PEO23 and SKOV3 cell lines (discussed in Section 1.7) - were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640; 218750, Gibco®) medium supplemented with 10% heat inactivated foetal bovine serum (FBS; 10270106, Gibco®) and 10,000 U/mL (1%) penicillin-streptomycin (P/S; 151401, Gibco®). Frozen stocks of these cell lines had previously been confirmed negative for mycoplasma and authenticated by Short Tandem Repeat profiling. The cell lines were grown in T-175 flasks (83.3912.002, Sarstedt), in a humidified incubator at 37°C, and 5% CO_2. Media were changed at least every two to three days or when required. When cells reached 80-90% confluence, they were washed twice with sterile phosphate buffer saline (PBS) and 0.05% trypsin-EDTA (1X) dissociation agent with phenol red (253000, Gibco®) was added and left for about 5 min or until the cells detached. Fresh medium with serum was added to stop the trypsinisation process and cells were either passaged into new culture vessels or centrifuged and used as required for further experiments.

2.1.1 Cell counting and seeding

Cells were detached by the use of trypsin as described above, and the cell suspension was centrifuged at 21°C, for 5 min at 1,000 g. The cells were re-suspended in fresh medium. To count the cells, 10 μL of the re-suspended medium was placed in the haemocytometer and an average cell number per mL was calculated. Cells were seeded into flat bottomed 96 well plates (Thermo Scientific) or 6 well plates at densities based on their known cell line proliferation rates.

2.1.2 Cell preservation and recovery

To preserve frozen stocks of the cell lines, cells were frozen down in a freeze mixture containing 10% dimethylsulphoxide (DMSO) with heat inactivated FBS and stored at -80°C in cryovials. For recovery of cells, the frozen cell suspension was rapidly defrosted, placed in routine medium and the cells were allowed to adhere to a T-25 flask. Medium was changed after 24 h to remove traces of DMSO and cells were allowed to grow.

2.1.3 Generation of multicellular tumour spheroids

When cells reached 70% confluence in T-175 flasks, they underwent tryspinisation and centrifugation at 21°C, for 5 min at 1,000 g. Cells were re-suspended using a syringe in fresh 10% FBS-containing medium. The solution was transferred to a 100 mL or 250 mL spinner flask. The spinner flasks were kept on a magnetic stirrer platform, in an incubator at 37°C and 5% CO_2, until spheroids reached a maximum size, typically after 5 days.

2.2 Cellular assays

2.2.1 Sulforhodamine B (SRB) assay

To assess the drug sensitivity of the ovarian cancer cell lines, the sulforhodamine B (SRB) assay was used (Figure 2.1). This assay quantifies the cellular protein content [344], which acts as an indirect measure of cell number. Cells were seeded as per section 2.1.1 in 100 µL of media. After 48 h, TKIs, mAbs or cytotoxic agents diluted in 100 µL of media were added to the wells (Table 2.1). The effects of 1 ng/mL EGF (AF-100-15, PeproTech) and 1 ng/mL HRG (396-HB/CF, R&D Systems) were also analysed. Day 0, untreated controls and vehicle controls were also included in each assay. Each assay was replicated at least three times.

	Drug	Catalogue No, Company	Concentration range *(μM)*
TKIs	Afatinib	S1011, Selleckchem	0.003-6
	Canertinib	S1019, Selleckchem	0.003-6
	Lapatinib	S2111, Selleckchem	0.003-6
	Neratinib	S2150, Selleckchem	0.003-6
	Sapitinib	S2192, Selleckchem	0.003-6
Cytotoxic agents	Carboplatin	S1215, Selleckchem	0.1-24
	Cisplatin	S1166, Selleckchem	0.05-12
	Paclitaxel	S1150, Selleckchem	0.00001-0.1
mAbs	Pertuzumab	*Kindly provided by Roche*	50 μg/mL
	Trastuzumab	*Obtained from Pharmacy, WGH*	50 μg/mL

Table 2.1: Drug inhibitors used and their concentration ranges

All TKIs and cytotoxic agents were bought in powder form and dissolved in DMSO, except for carboplatin which was solubilised in sterile water. The mAbs were supplied in prepared stock solutions.

Cells were fixed after they had been incubated for 24 h - 120 h, by the addition of 50 μL per well of cold 25% Trichloroacetic acid (TCA), and left for 1 h at 4°C. Afterwards, the plates were washed ten times with cold water, and allowed to dry in the oven at 50°C. Once dry, cells were stained with 50 μL of SRB dye solution and incubated for 30 min at room temperature. The SRB dye was washed four times with 1% acetic acid, and left to dry. The cell-bound SRB dye was solubilised in 150 μL of 10 mM Tris solution, at pH 10.5, followed by the measurement of optical density (OD) at 540 nm in a BP800 Biohit Microplate Reader. Results were processed in an Excel sheet.

After reading the plates in the Microplate reader, a mean of the values of wells with the same drug concentrations was calculated. The growth as a percentage of growth of control was determined through the following formula: % Growth = B−A/C−A × 100, where A = A540 nm before treatment, B = A540 nm after treatment with drugs, and C = A540 nm after medium control.

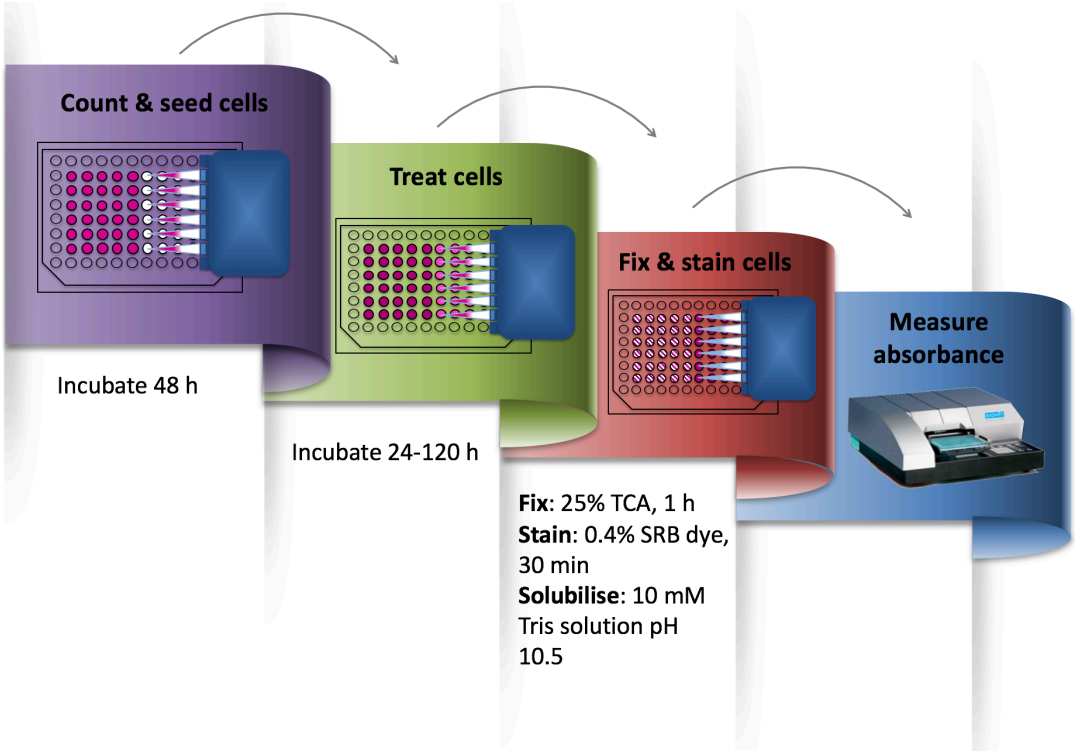


Figure 2.1: Pictorial depiction of the SRB proliferation assay

2.2.1.1 Combinatorial treatment

To assess the effects of a combination of drugs on cell proliferation, combinatorial experiments were carried out. After 48 h cell incubation in 96 well plates, the IC_{20} and IC_{30} values of cytotoxic drugs were combined with eight times the IC_{50} value of neratinib or lapatinib, followed by up to eight doubling dilutions added to the wells, either simultaneously or 2 h pre-treatment with the TKIs. The cells were fixed after 120 h and the SRB assay was followed as in section 2.2.1.

The measurable quantity of drug combination effects, combination index (CI) was calculated using CompuSyn Software [345]. The growth effects of the single agents and the combined agents were computed with values ranging from 0 to 1,

corresponding to the drug concentrations used. The CompuSyn Software analyses the median-drug effects, which was originally described by Chou and Talalay [346]. This calculation assumes that the drugs, alone or in combination, would result in a sigmoidal concentration-response curve, and considers the potency of each drug as well as the shape of the concentration-response curve. The values which did not cause any growth inhibition, and the concentrations which caused complete cell kill were disregarded, as they decrease the sensitivity of the calculation. The CI values for each combination concentration indicated Synergism (CI<0.9), Additive Effect (CI: 0.9-1.1) and Antagonism (CI>1.1).

2.2.1.2 IC_{50}

The half maximal inhibitory concentration (IC_{50}) is the concentration of inhibitor which is capable of halving the cell number. The IC_{50} value for each drug in different cell lines was calculated from sigmoidal concentration response curves using GraphPad Prism version 8 to give an indication of the potency of a drug.

2.2.2 Migration (scratch) assay

Cells were seeded in 6-well plates at a density of $1x10^6$ - $2x10^6$ cells per well in routine medium. When the cells reached 100% confluence (generally after 24 h), a perpendicular line was scratched across each well using a 200 µL pipette tip. Plates were washed in PBS until free floating cells had been removed and a clean well-defined scratch was produced. The routine medium was replaced by medium containing 0.1% FBS and 1% penicillin-streptomycin. Some wells were used for controls (DMSO or media only), while others contained drug treatments, with or without growth factors.

Photomicrographs were taken at time 0 h, and at regular time intervals thereafter using the x2.5 lens objective (Figure 2.2a). Marks were drawn on the lids so the same area would be photographed at every time point. Results were analysed using ImageJ software, based on the percentage area migrated relative to the respective 0 h area.

The percentage that the cell lines migrated was calculated by subtracting the scratch area at 24 h from the scratch area at 0 h as illustrated in Figure 2.2b.

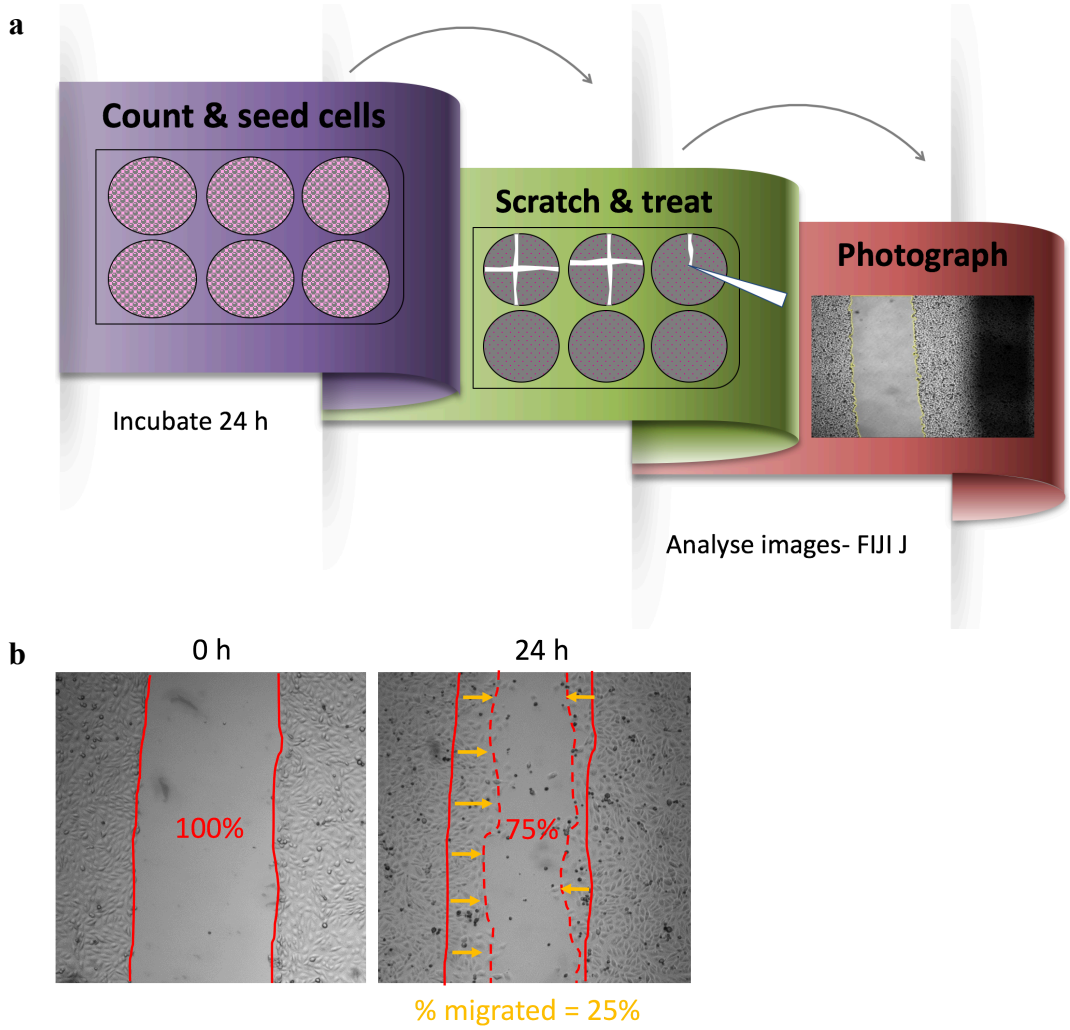


Figure 2.2: Migration (scratch) assay protocol

(a) Pictorial depiction of the migration (scratch) assay protocol. (b) Example of how the percentage area migrated was calculated. The area remaining after migration was calculated as a percentage from the total area migrated relative to 0 h.

2.2.3 Adhesion assay

Cells were grown in 100 mm petri dishes, until they reached 70% confluence. Cells were then serum starved for 2 h and treated with 0.1 µM neratinib for 15 min. Cells were then re-suspended in serum free media containing the respective treatments. Cells were seeded in large numbers in uncoated, collagen coated (C7661, Sigma Aldrich), fibronectin coated (F1141, Sigma Aldrich) or laminin coated (L2020, Sigma Aldrich) plates. For collagen coated plates, 1 µg/cm^2 of collagen was diluted in sterile PBS,

then added to the wells with minimal volume of solution and was left overnight at 4°C. For fibronectin coated plates, 1 μg/cm^2 of fibronectin was diluted in sterile PBS, then added to the wells with minimal volume of solution, and allowed to air dry for 45 min at room temperature. For laminin coated plates, 1 μg/cm^2 of laminin was diluted in sterile PBS, added to the wells with minimal volume of solution, left for 2 h at 37°C and washed once with sterile PBS.

The medium in the wells was changed after 1 h to normal medium and cells were fixed, and underwent the SRB assay protocol as per section 2.2.1. Results of the coated plates were then normalised to uncoated plates at each time point.

2.2.4 Collagen invasion assay

Cells were grown in 3D spheroids as described in section 2.1.3. When spheroids reached a maximum size, they were removed from spinner flasks and gently transferred to a petri-dish using a 25 mL serological pipette.

A collagen mix was prepared as per Table 2.2, with or without treatment, and all contents were kept on ice before and after preparation. A single spheroid was taken up in 500 μL collagen mix solution, and placed centrally into a well in a 24-well plate (Figure 2.3a). The spheroids were incubated at 37°C, 5% CO_2 for 1 h to allow the collagen solution to polymerise. Then, the polymerised gel solution was released from the edges and 500 μL media solution with or without treatment was added to each well respectively.

Content	Specifications	Volume (mL)
Collagen	Ready-bought Cellmatrix type I-A (Alphalabs)	2.5
Acetic Acid (0.1%)	50μL of 100% acetic acid to 50 mL dH_2O	4.5
FBS	Cell culture FBS	1
NaOH (0.22M)	0.87993g NaOH (Sigma) into 100 mL dH_2O	1
10x DMEM	One vial DMEM (Sigma) into 100mL dH_2O	1

Table 2.2: Contents of the collagen mix, required to make 10mL of solution
The materials were made up outside the cell culture hood and were then passed through an Acrodisk 25 mm syringe filter in the cell culture hood.

Photomicrographs were taken using the x2.5 objective lens at time 0 h, 24 h and every 24 h thereafter, for up to 120 h. Results were analysed using ImageJ software, based on the percentage area invaded relative to the respective 0 h (Figure 2.3b).

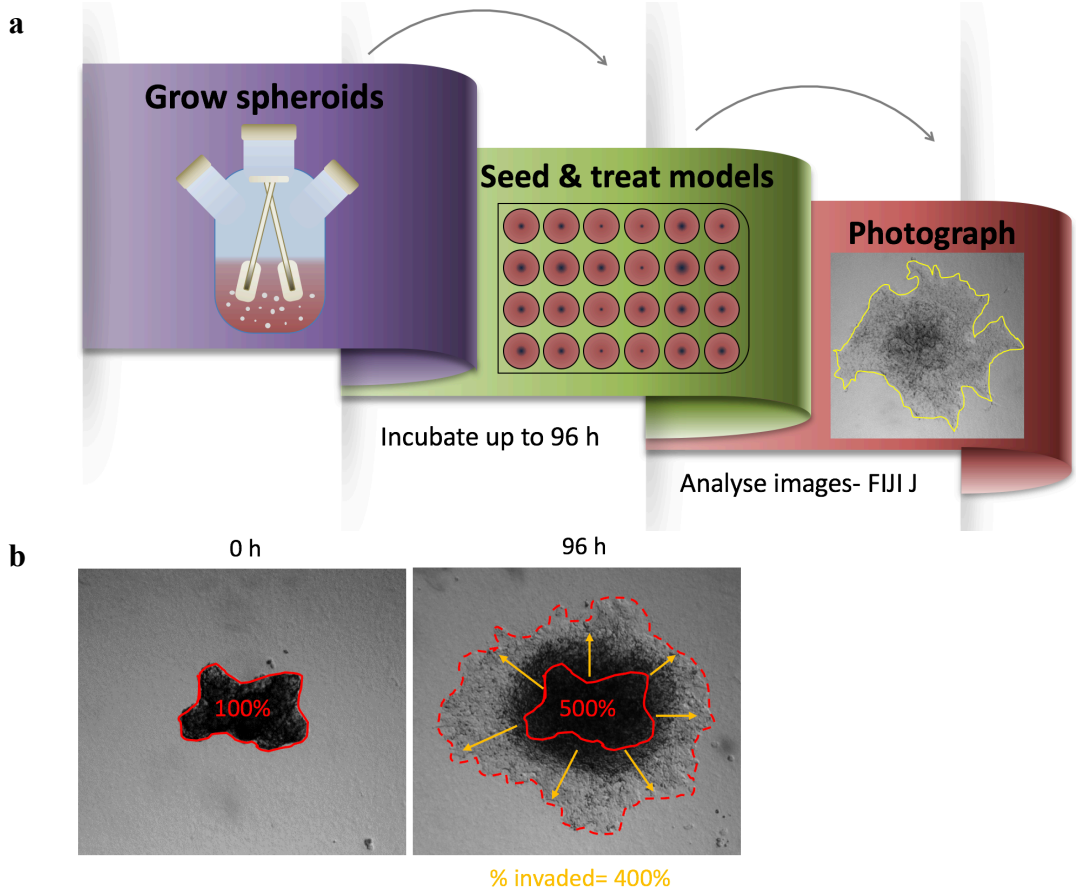


Figure 2.3: Invasion assay protocol

(a) *Pictorial depiction of the invasion assay protocol. (b) Example of how the percentage area invaded was calculated. The area invaded was calculated by subtracting the 0 h area from the area at each time point and presented as a percentage.*

2.3 Protein expression studies

Protein expression in the cell lines was measured using the western blot technique following protein acquisition (Figure 2.4). Western blotting separates specific proteins in a sample based on molecular weight. The process allows for detection and quantification of proteins of interest.

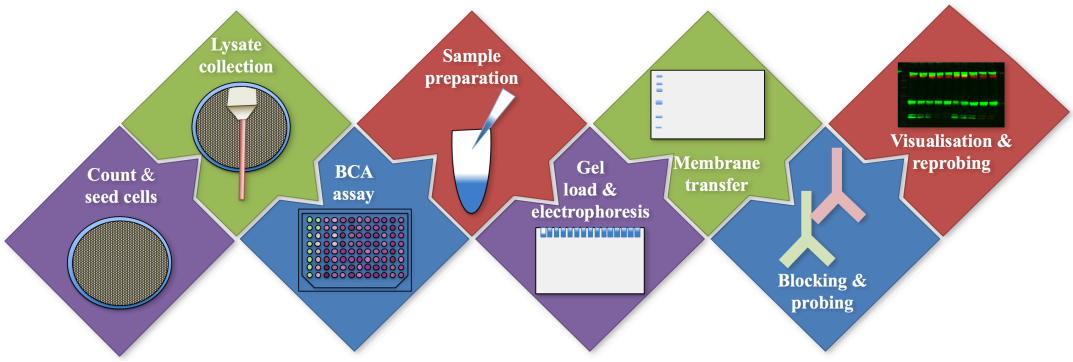


Figure 2.4: Pictorial depiction of the western blot protocol

2.3.1 Protein lysate collection

Cells were grown in 100 mm petri dishes (Scientific Laboratory Supplies) or 6 well plates, in an incubator at the previously described conditions. Once they reached 80% confluence, they were washed twice with ice cold PBS and left on ice to preserve the proteins. 200 µL or 100 µL of the lysis buffer (Table 2.3) was pipetted onto 100 mm petri dish or 6 well plates respectively, and cells were scraped. After 10 min, the lysate was collected and placed in pre-labelled eppendorfs, and centrifuged at 13,000 g for 6 min at 4°C. The supernatant was aliquoted by placing 10 µL in one eppendorf (for protein quantification) and the remaining supernatant into a 1.5 mL eppendorf for storage. Samples were stored at -70°C.

Lysis buffer additives	Catalogue No, Supplier
1 complete protease inhibitor tablet	11836153001, Roche
100 µL phosphatase inhibitor cocktail 2	P5726, Sigma Aldrich
100 µL phosphatase inhibitor cocktail 3	P0044, Sigma Aldrich
50 µL Aprotinin	A6279, Sigma Aldrich
100 µL TritonX-100	X100, Sigma Aldrich

Table 2.3: Components of the lysis buffer

The lysis buffer was made up of 50 mM Tris, 5 mM EGTA, 150 mM NaCl. The additives in the table were added to 10 mL of lysis buffer immediately before use.

2.3.2 Bicinchoninic acid (BCA) assay

This assay was used to determine the protein concentration in each sample. Lysates were placed on ice and a standard curve was prepared using eight known concentrations of protein standard (P0914, Sigma Aldrich), as per Table 2.4. Five µL

from each protein sample was added to 45 µL of distilled water in borosilicate tubes. One mL of BCA solution (1 mL of copper sulphate solution (C2284, Sigma Aldrich), and 49 mL BCA solution (B9643, Sigma Aldrich)) were added to all protein samples and standards. These were incubated at 60°C for 15 min. In a 96 well plate, 200 µL of each solution was pipetted in duplicate, and absorbance was measured at 540nm using a BP800 Biohit Microplate Reader. Sample protein concentration was determined from the protein concentration plot.

Tube	Final Concentration (µg/mL)	Volume of 1 mg/mL Protein Standard (µL)	Volume of Distilled H_2O (µL)
A	0	0	50
B	50	2.5	47.5
C	75	3.75	46.25
D	100	5	45
E	250	12.5	37.5
F	500	25	25
G	750	37.5	12.5
H	1000	50	0

Table 2.4: Dilutions of the protein standard for the protein standard plot

2.3.3 Gel electrophoresis and transfer onto PVDF membrane

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate proteins according to their molecular weight. SDS polyacrylamide gels were set up using the Bio-Rad mini-Protean equipment using resolving and stacking gels (Table 2.5).

	7.5% Resolving gel (mL)	3.6% Stacking gel (mL)
30% Acrylamide	10	3.6
1M Tris, pH 8.85	15	-
0.375M Tris, pH 6.8	-	10
10% SDS	0.4	0.3
dH_2O	14.6	16
TEMED	0.1	0.1
10% APS	0.1	0.1

Table 2.5: Volumes of solutions required to produce 4 mini-gels
Resolving gel percentage can be altered depending on the molecular sizes of the proteins to be detected.

Forty µg protein samples (containing the predetermined amount of protein lysate from BCA, lysis buffer and 5X loading buffer), were denatured at 95°C for 5 min, and loaded into the gels. Pre-stained protein marker (13953, Cell Signaling) was also loaded to aid identification of protein bands. Gels were electrophoresed through a stacking gel at 80 V for 15 min, and through the resolving gel at 150 V for about 90 min with the aid of a running buffer (Table 2.6).

Afterwards, proteins were transferred onto a methanol-activated Immobilon-P polyvinylidene fluoride (PVDF) Transfer Membrane (Immobilon). Transfer was performed at 100 V for 90 min in ice-cold transfer buffer (Table 2.6) at 4°C.

	Running buffer	**Transfer buffer**
Tris base	6.06 g	6.06 g
Glycine	28.84 g	28.84 g
dH_2O	2000 mL	2000 mL
10% SDS	20 mL	-

Table 2.6: Contents of running buffer and transfer buffer enough for 4 mini-gels

2.3.4 Probing and visualisation

The transfer membrane was blocked for 1 h at room temperature with 1:1 PBS/ Odyssey blocking buffer (Odyssey) to prevent non-specific binding. Afterwards, the membrane was left overnight in primary antibody solution (Table 2.7) at 4°C. Then it was washed three times in PBS-0.1% Tween 20 to remove excess antibody. The membrane was subsequently incubated for 45 min, in the dark, and at room temperature with secondary fluorescent antibodies IRDye 680LT and IRDye 800CW. The membrane was then washed with PBS-0.1% Tween 20 three times then again with PBS for three times (each wash was 5 min in duration on a rocker).

Primary Antibodies	Catalogue No	Dilution	Observed MW (kDa)	Species	Secondary antibodies
EGFR	4267	1:1000	175	Rabbit	Anti-rabbit IR Dye 680LT (1:10000)
p-HER2 (Tyr1221/1222)	2243	1:1000	185		
p-Akt (Ser473)	9271	1:1000	60		
ERK	9102	1:1000	42, 44		
p-HER3 (Tyr1289)	2842	1:1000	185		
HER3	12708	1:1000	185		
HER4	4795	1:500	180		
p-EGFR (Ser1047)	24918	1:2000	175	Mouse	Anti-mouse IR Dye 800CW (1:10000)
HER2	2248	1:1000	185		
Akt	2920	1:1000	60		
p-ERK (Thr202/Tyr204)	9106	1:1000	42, 44		
Beta-actin	8227	1:8000	42		

Table 2.7: The concentrations of the antibodies used were according to the manufacturer's product usage information

All primary antibodies were from Cell Signaling Technology, except for p-EGFR and beta-actin which were from Abcam. The secondary antibodies are from Licor Odyssey. The primary antibodies were diluted in 1:1 PBS/ Odyssey blocking buffer solution, while the secondary antibodies were in 1:1 PBS/Odyssey Blocking Buffer containing 0.001% SDS. The antibodies selected for western blotting had all been previously validated for use within the laboratory and demonstrated banding at the expected molecular weight. Where MW means molecular weight.

Proteins were visualized using the Odyssey Infrared Imaging System (Licor). Odyssey is equipped with two infrared channels for direct fluorescence detection at 680LT and 800CW. The images were viewed and intensity of signal quantified using Image Studio Lite software (Licor).

2.4 Effects of TKIs on signalling pathways

Cells were seeded in 6-well plates at a density of $2x10^6$ cells per well, in routine media. After 24 h, cells were washed twice with warm PBS, and media containing 0.1% FBS and TKIs was added to each well. The concentrations of TKIs were as follows: 0.08 µM afatinib, 1 µM canertinib, 0.1 µM neratinib, 1.5 µM lapatinib and 1 µM sapitinib, based on clinically relevant concentrations (concentrations specified in Table 1.3). The

cells were incubated with drugs for 24 h. Cells were treated with 1 ng/mL EGF or 1 ng/mL HRG 30 min prior to lysate collection as described in section 2.3.1.

2.4.1 Time course

Cells were seeded in 6-well plates at a density of $2x10^6$ cells, in routine media. After 24 h cells were washed twice with warm PBS. Medium with 0.1% FBS and without any other added growth factor was aliquoted into the wells and incubated for 2 h. The media was then replaced with RPMI 1640 media containing 0.1 μM neratinib and/or 1 ng/mL EGF and/or 1 ng/mL HRG. Controls were seeded on separate plates. Cells then underwent lysis as described in section 2.3.1 at 0, 5, 10, 15, 30 and 60 min time points.

2.5 Immunostaining

Immunostaining encompasses a set of techniques used to visualise the precise location of proteins or antigens within the cell.

2.5.1 Immunohistochemistry of tissue microarrays

The TMAs used in this study were obtained from mouse xenografts, which were treated with pertuzumab, trastuzumab or the combination over a period of time [232]. Tissues were formalin-fixed and paraffin-embedded, with triplicate cores cut and mounted onto TMA slides. Sections of the TMAs were cut and placed onto slides (Histology Laboratory, University of Edinburgh).

The TMAs were initially deparaffinised by placing them twice in xylene for 5 min each time, then rehydrated by placing them for 2 min each time in decreasing concentrations of ethanol (99%, 99%, 80% and 50%) and afterwards, washed in running tap water. The sections were antigen retrieved by heating sodium citrate buffer at pH6 (18 mL 0.1 M citric acid, 82 mL 0.1 M sodium citrate, 900 μL dH_2O) for 10 min in a pressure cooker and then, placing the slides in the heated buffer and cooking for 5 min in a microwave. The slides were then allowed to cool. Afterwards, they were washed twice in PBS-0.1% Tween20 solution followed by the addition of 500 μL of

3% H_2O_2 onto each coverslip and incubation at room temperature for 10 min. After washing with PBST, 300 µL of blocking buffer (DAKO) was pipetted onto each coverslip and incubated for 10 min at room temperature in a humid environment. Following this incubation, 200 µL of primary antibody diluted in DAKO antibody dilution solution was pipetted onto the coverslips and incubated overnight at 4°C. Negative controls consisted of adding 200 µl DAKO antibody dilution solution without adding any primary antibodies to the coverslips. Coverslips were then washed twice with PBST and 6 drops of Envision labelled polymer (secondary antibody) were added and incubated for 30 min at room temperature. A 1:50 chromophore and substrate solution (DAKO) was prepared and 250 µL was added to each coverslip and left for 10 min. Coverslips were then assembled in a rack, counterstained in haematoxylin for 1 min and gently washed in water. Then, they were placed in Scott's tap water substitute (STWS) for 30 s, washed gently with water and placed in DE-HYD 50%, DE-HYD 80% solutions consecutively for 30 s. Later, they were placed twice in DE-HYD 100% solution for 2 min each time, and dehydrated in Xylene for 10 min. DXP mountant was used to fix coverslips onto slides, and the slides were left to dry overnight. Slides were visualised using a Hamamatsu Nanozoomer XR.

Primary antibodies	Catalogue No, Supplier	Dilution	Species
CEBPG	NBP1-89742, Novus	1:20	Rabbit
DDIT4L	12094-1-AP, Proteintech	1:50	Rabbit
RALGDS	NBP2-30584, Novus	5 µg/ml	Rabbit
SPRY2	H00010253-M01, Novus	1:50	Mouse

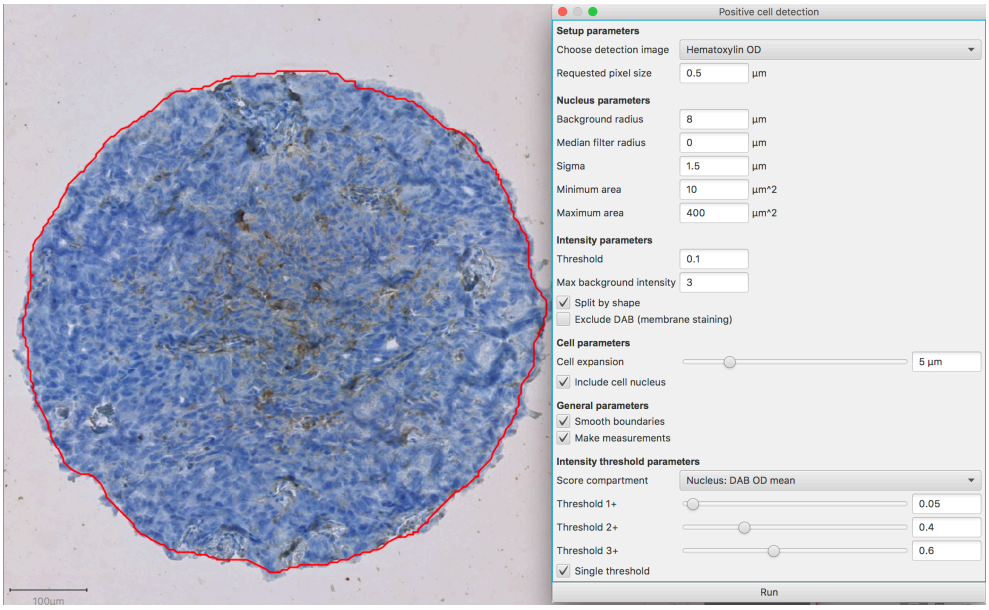
Table 2.8: Details of the primary antibodies used on the TMAs

2.5.2 Analysis of TMA

The TMAs were analysed using QuPath Software [347], a digital pathology image analysis programme, which recognises the areas of staining and outputs a percentage of positively stained cells from the total number of cells. To analyse, the area was initially selected and the threshold for detection was specified (Figure 2.5a). The

"Positive %" was used as a measure of protein presence within the sample (Figure 2.5b). This "positive %" score was multiplied by manually analysing the intensity of staining from 1 to 3, where 1 represented weak staining, 2 represented moderate staining and 3 signified strong staining.

a



b

Figure 2.5: QuPath software screenshots

(a) selection area and optimisation of parameters, (b) percentage positive detection.

2.6 RNA interference

2.6.1 Preparation of SiRNA

Individual ON-TARGETplus SiRNA oligonucleotides targeting human EGFR were used (Table 2.9) as well as a non-targeting scrambled SiRNA (D-01810-01-20, Dharmacon) control. ON-TARGETplus SiRNA was chosen as off-target effects are reduced by up to 90%. SiRNA solutions were prepared at 20 μM stock solution, by adding 1x SiRNA buffer solution (dilution of 5x SiRNA buffer (Dharmacon) into RNase-free water (Dharmacon)). The solution was left for 30 min on an orbital shaker at room temperature. A NanoDrop Spectrophotometer ND1000 was used to identify the concentration of the reconstituted SiRNA solution. The individual oligonucleotides specified before were used as they produced the best knock-downs upon optimisation experiments.

Target	Target Sequence	Catalogue No
EGFR (individual)	GUAACAAGCUCACGCAGUU	J-003114-12
EGFR (individual)	CAGAGGAUGUUCAAUAACU	J-003114-13

Table 2.9: A list of SiRNA oligonucleotides used in this study
The sequence they target and catalogue number are detailed. All SiRNAs used in this study were purchased from Dharmacon.

2.6.2 SiRNA transfection

Cells were seeded in 96 well plates or 6 well plates to assess the effects of EGFR knockdown on proliferation and migration respectively. RPMI 1640 media containing 10% FBS and no P/S was used to seed the cells and these were seeded at densities that would achieve 60% confluence the following day. Cells were transfected 24 h after seeding, following manufacturer's protocol, as illustrated in Figure 2.6a for proliferation assays, and Figure 2.6b for migration assays. Briefly, SiRNA solution or transfection reagent were incubated with serum-free and P/S free media for 5 min at room temperature. They were then combined and incubated for an additional 20 min

at room temperature. The solutions were then pipetted into their respective wells. Each experiment contained three controls; an untreated, a mock and a non-targeting control.

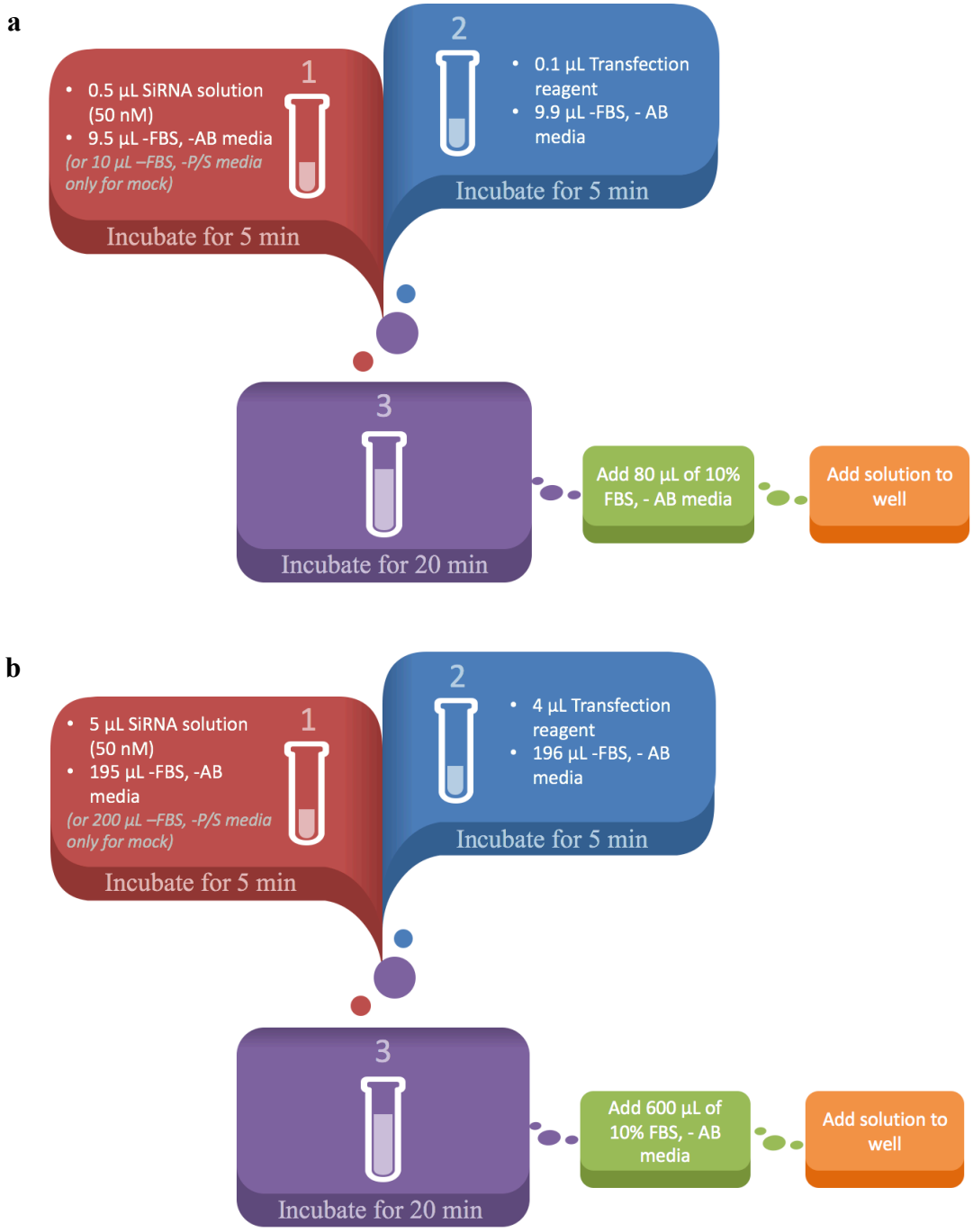


Figure 2.6: Figure illustrating the protocol for SiRNA transfection
(a) *96 well plate, (b) 6 well plate. The values represent the volumes required per well.*

2.7 Generation of stable cell lines overexpressing HER

2.7.1 Antibiotic optimisation

To generate stable cell lines that would express the gene of interest, the minimum concentration of selection antibiotic, in this case Geneticin® (G418; 11811031, Thermo Fisher Scientific) that would kill untransfected cells was determined. Cells were seeded in 96 well plates at 2,000 cells per well, in P/S free media. After 24 h, the medium was changed to medium containing G418 at concentrations ranging from 100 – 900 µg/mL. A control consisted of routine medium with P/S. The medium was changed every 3-4 days with the same concentrations of G418. The cells were observed daily under the microscope. After 2 weeks of culturing, the minimum concentration that killed all the cells in each cell line was established.

2.7.2 DNA plasmid extraction

HER2 WT (Addgene Plasmid #16257) and pcDNA3-HER3-DMVF-181-R (Addgene Plasmid #87621) were bought from Addgene as agar stabs. A 200 µL pipette was used to transfer a small amount of bacteria from the stabs and this was transferred to 3 mL Lysogeny broth (LB) with ampicillin in falcon tubes (Figure 2.7). These were left to incubate at 37°C on a shaker for 6 h. Later the bacteria growing in falcon tubes were transferred to 200 mL of LB without ampicillin in conical flasks and were allowed to grow at 37°C on a shaker overnight.

The following day, the media suspension was centrifuged for 10 min at 6,000 g. The supernatant was discarded and the bacterial pellet was re-suspended in 10 mL of R1 buffer (12162, QIAGEN MaxiPrep). Ten mL of R2 was added and mixed vigorously until the solution was blue and left to stand for 5 min. Then 10 mL of pre-chilled R3 was added and mixed until the solution turned colourless. The solution was centrifuged at 4°C for 1 h at 14,000 g. In the meantime, QIAGEN tip 500 was equilibrated by adding 10 mL of Buffer QBT, and allowing it to flow through by gravity. The centrifuged supernatant was added to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed twice with 30 mL of Buffer QC and

allowed to flow through by gravity. Afterwards, the DNA was eluted using 15 mL Buffer QF into a fresh 50 mL tube. The DNA was then precipitated by adding and mixing 10.5mL isopropanol to the eluted DNA. The solution was centrifuged for 1 h at 4°C and 15,000 g. The DNA pellet was washed with 5mL 70% ethanol at room temperature and centrifuged for 10 min at 15,000 g. The supernatant was discarded and the DNA pellet was left to air dry for 1 h. The dry DNA was re-suspended in 500 µL RNA-free water.

The quality and quantity of the DNA was assessed using Nanodrop™ Spectrophotometer (Thermo Scientific). The spectrophotometer was firstly blanked, then 1 µL of the sample solution was measured for the concentration (ng/µL) and purity using the A_{260}:A_{280} and A_{260}:A_{230} ratios. An A_{260}:A_{280} ratio of around 2.0 is considered pure.

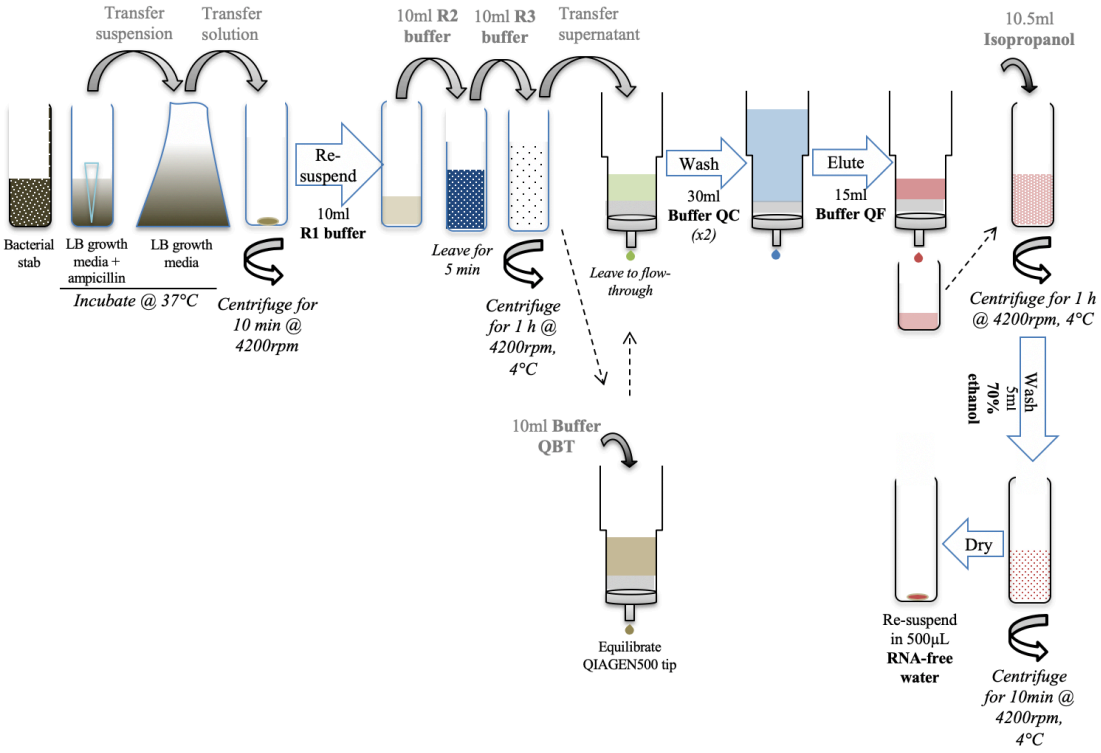


Figure 2.7: Pictorial depiction of the protocol followed to extract DNA from plasmids

2.7.3 Transfection of plasmids into cells

Over 1x10^5 cells were seeded in 500 µL of antibiotic-containing medium in 24 well plates. After 24 h incubation, the cells were transfected with 1:2 ratio of plasmid DNA

solution to Lipofectamine 2000 transfection agent (116680, Thermo Fisher Scientific), as illustrated in Figure 2.8. This was done by separately diluting 1 µg of plasmid solution and 2 µL Lipofectamine 2000 transfection agent into 50 µL antibiotic free Opti-MEM medium. These two solutions were left to incubate at room temperature for 5 min. They were then combined and left to further incubate at room temperature for 20 min. The final transfection solution was gently added into the well. After 6 h, the medium of the cells was changed to fresh medium without antibiotics. After 24 h, the cells were trypsinised and transferred to 6 well plates, and G418 antibiotic was added to the cells at concentrations specified in Table 2.10, determined from antibiotic optimisation, detailed in section 2.7.1. The G418-containing medium was changed twice weekly. When cells reached 70% confluence, they were transferred to 24 well plates in very low cell dilutions. When colonies formed, they were transferred to 6 wells plates, and finally to T-175 flasks for routine tissue culture. Cells were always supplemented with G418 to maintain selectivity.

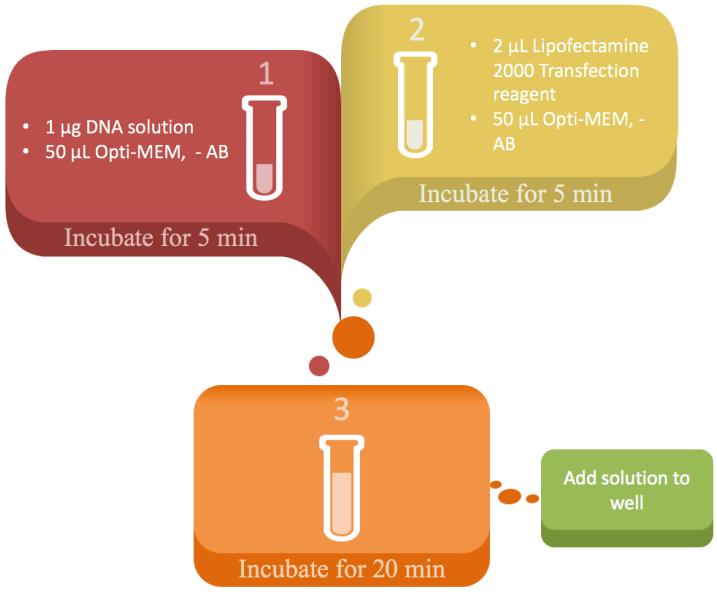


Figure 2.8: Figure illustrating the protocol for plasmid transfection in a 24 well plate
The values represent the volumes required per well.

Cell line	PEA1	PEA2	PEO1	PEO4	PEO14	PEO23
[G418] (µg/mL)	500	200	300	400	200	400

Table 2.10: Concentrations of G418 used for each cell line
The G418 antibiotic was purchased in powder form and dissolved in sterile distilled water according to the manufacturer's instructions.

2.8 Gene expression analysis

2.8.1 Sample collection and RNA extraction

Cells were seeded in 60 mm plates at a density of $2.5x10^6$ and incubated for 24 h. Cells were incubated in decreased serum conditions (0.1% FBS) for 6 h, with or without 0.1 µM neratinib. 10 ng/mL HRG was added to HRG control and HRG + 0.1 µM neratinib treatment for 90 min. Cells were then washed twice and trypsinised. The samples were centrifuged at 3,000 g for 5 min at 21°C. The cell pellets were re-suspended in 500 µL PBS, transferred to RNA-free microcentrifuge tubes and centrifuged for another 5 min at maximum speed. The supernatant was removed and cell pellet was stored at -80°C for RNA extraction.

The QIAshredder (79654, Qiagen) and RNeasy Plus Mini kit (74134, Qiagen) were used for the extraction. The manufacturer's protocol was followed, as illustrated in Figure 2.9. Briefly, the defrosted cell pellet was lysed and homogenised in 600 µL of RLT Buffer Plus. This was transferred to a QIAshredder assembled tube and centrifuged for 2 min at high speed. The flow-through was transferred to an assembled genomic DNA (gDNA) eliminator tube and centrifuged for 30 s. A volume of 600 µL of 70% ethanol was added to the flow-through, transferred and centrifuged for 15 s in two lots. The flow-through was discarded, and the column was washed with 700 µL Buffer RW1 (centrifuged for 15 s), and twice with Buffer RPE containing 80% ethanol (centrifuged for 15 s and then 2 min). The column was transferred to a new 2 mL collection tube and centrifuged for an additional 1 min to dry the spin column's membrane. The column was then transferred to a 1.5 mL collection tube and 40 µL RNase-free water was added to the membrane and centrifuged for 1 min to elute the RNA.

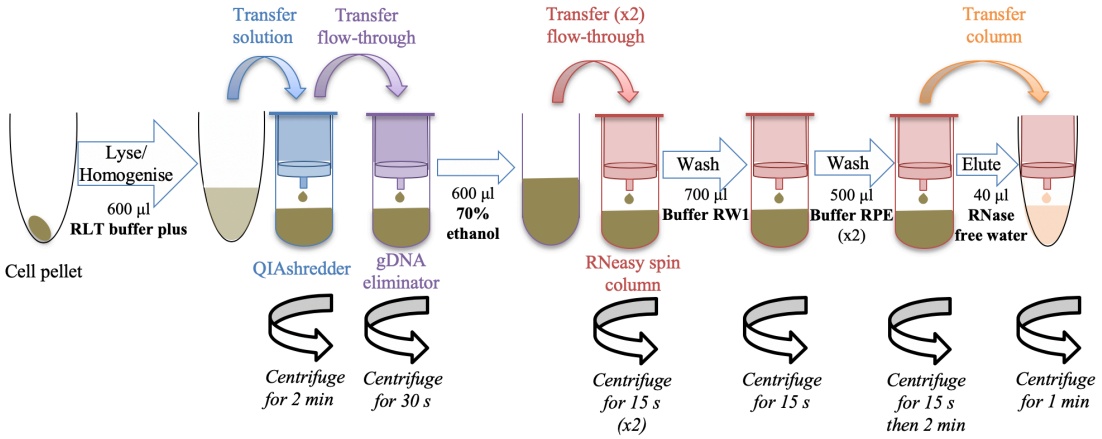


Figure 2.9: Pictorial depiction of the protocol for RNA extraction using QIAshredder and RNeasy Plus Mini kits (by Qiagen)

Centrifugation was carried out at high speed. All buffers were supplemented with the kit.

The quality and quantity of the RNA nucleic acid was assessed using a Nanodrop™ Spectrophotometer (Thermo Scientific). The spectrophotometer was firstly blanked, then 1 µL of the sample solution was measured for the concentration (ng/µL) and purity using the A_{260}:A_{280} and A_{260}:A_{230} ratios. An $A_{260:280}$ ratio of around 2.0 is considered pure.

2.8.2 mRNA sequencing and gene expression analysis

Targeted whole transcriptome mRNA sequencing was performed using QuantSeq Lexogen (Edinburgh Clinical Research Facility, University of Edinburgh). The raw data obtained were Log2 transformed and quantile normalised.

Differential gene expression analysis by Rank Product was conducted using TM4 MeV (multiple experiment viewer) software [348]. Rank product was used because it is a strong tool to analyse the differently expressed genes in small data sets, with reliable statistical power, and robustness against inconsistent changes, such as single outliers [349]. Gene enrichment of the differently expressed genes was performed online using DAVID[3] and Gene Ontology (GO) was used to determine pathways and molecular function of the differently expressed genes [350]. Gene data were mean centred,

[3] DAVID online resource, available from: https://david.ncifcrf.gov

average linked and clustered on Cluster 3.0 [351] and viewed in the form of heatmaps on Java Treeview software [352].

Publicly available gene expression datasets were mined to compare and contrast with the gene expression results obtained from this study. To do this, GEO datasets from the US National Library of Medicine National Institutes of Health website[4] were accessed, and data sets containing gene expression of several cancer types treated with TKIs, mAbs or growth factors were downloaded.

2.9 Statistical analysis

Statistical tests were performed using GraphPad Prism version 8. Graphical data expressed as mean ± standard error were also generated using GraphPad Prism. The one-way or two-way ANOVA multiple comparison test was used to compare more than two groups, while the unpaired-sample t-test was used to compare data between two groups. Correlations between two variables were assessed using Spearman's correlation.

[4] Geo Datasets, available from: https://www.ncbi.nlm.nih.gov/gds/

3 Effect of HER-targeted therapy on ovarian cancer cell functionality

Initial investigations sought to assess the effectiveness of HER-targeted inhibitors on HGSOC cellular functionality. The HER-targeted inhibitors examined were five TKIs and two mAbs, which allowed comparisons between inhibitors with different modes of actions. The TKIs investigated were pan-HER inhibitors, which, unlike the first generation TKIs gefitinib and erlotinib, do not require specific HER family mutations to elicit their effects [259, 353]. The TKIs studied were afatinib, canertinib, lapatinib, neratinib and sapitinib, which differentially target HER family members and offer varied pharmacokinetic profiles [147]. Trastuzumab and pertuzumab were the mAbs investigated in this study, which are both currently used for the treatment of breast cancer [354]. A panel of HGSOC platinum-resistant and platinum-sensitive pairs of cell lines was used to investigate the effects of these inhibitors on cellular proliferation, adhesion, migration and invasion. This is a novel approach to the functional investigations of these TKIs, as to our knowledge, no other study to date has investigated their use in platinum-sensitive and resistant pairs of ovarian cancer cell lines.

HER family protein expression levels across the cell line panel were first investigated in order to assess whether the potency of the inhibitors was related to HER expression levels. The anti-proliferative effects of the TKI and mAb panel of drugs were studied to assess the sensitivity of the cell lines to these agents, and in order to potentially link these effects to HER family expression or sensitivity to platinum-based therapy.

In the clinical scenario, most patients will receive combination therapy as it is unlikely that a patient would fully benefit from monotherapy only. In ovarian cancer for instance, carboplatin and paclitaxel are used in combination. To date, there are no approved drug combinations involving HER-targeted TKIs in HGSOC. The intentions of these experiments was to assess whether combining cytotoxic agents with TKIs would lead to lower concentrations being required for a sufficient anti-proliferative effect and explore novel combinations that could be considered for use in HGSOC.

Cancer is characterised by its ability to invade, metastasise and adhere to organs distant from the primary tumour. Downstream components of the HER pathway have been implicated with increased progression potential in ovarian cancer [355]. Hence, the objective was to evaluate the role of TKIs and mAbs for their ability to inhibit the progression of cancer, and to assess which HGSOC phenotypes were responsive to these agents.

3.1 HER expression in the panel of cell lines

The panel of six cell lines investigated in this study were initially characterised for their expression of the four HER receptors, as these receptors are the targets for the drugs under investigation. The cells were grown in 10% FBS media, without other additives, and lysates were collected once they reached 70% confluence and proteins detected by western blot.

EGFR was expressed by all cell lines to different extents (Figure 3.1). The PEO1 cell line expressed the highest level of EGFR, while PEO14 had the least EGFR expression. HER2 was expressed in five of the six cell lines, with PEA2 expressing the greatest level, while HER2 expression in PEA1 was not detectable. The platinum-resistant cell lines expressed over double the amount of HER2 than their respective platinum-sensitive cell lines. HER3 was expressed in all cell lines, with highest expression in PEO1, whereas there was minimal expression in PEA1. HER4 was only expressed in the PEA2 and PEO4 cell lines.

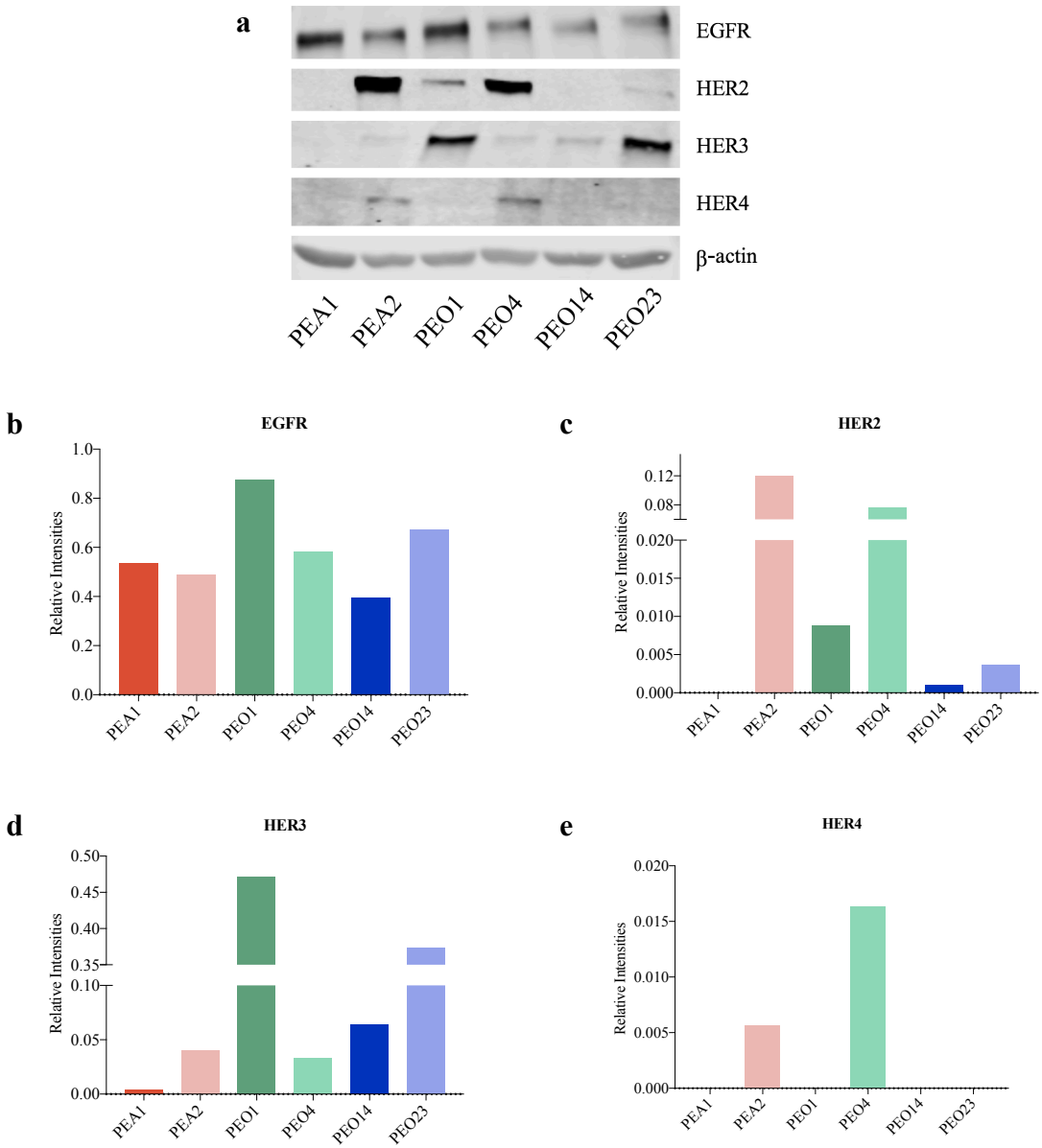


Figure 3.1: HER family expression across three pairs of HGSOC cell lines

(a) Protein expression of the 4 HER receptors was detected by western blot. The intensity of the band was measured using Image Studio Lite software, and each HER intensity band was normalised to the β-actin control. The relative intensities for each cell line are presented in bar graphs (b) EGFR, (c) HER2, (d) HER3 and (e) HER4. This experiment was repeated once and the loading control is a representative to the other membranes.

3.2 Effects of the agents on cell growth

The effect of the drugs on proliferation was investigated in all six cell lines; PEA1, PEA2, PEO1, PEO4, PEO14 and PEO23 using the SRB assay, following a 120 h incubation with the cells. A range of concentrations was employed, based on initial optimisation of the drug response in all cell lines.

3.2.1 Cell proliferation of the cell line panel

This experiment was undertaken to assess the growth differences of the cell line panel over a 120 h period. The cells were seeded to achieve 30% confluence after 24 h. They were supplemented with routine media, containing 10% FBS and P/S, and fixed at 24 h intervals. To quantify the cells, the SRB proliferation assay was used. The OD value at 0 h of the respective cell line was subtracted at every time point to obtain the increase in OD over time and results are presented as percentage growth increase. Figure 3.2 shows that the PEA1-PEA2 pair have the highest proliferation rate amongst the cell lines, whilst PEO14-PEO23 pair have the lowest proliferation rate. The platinum-resistant cell line PEO4 grew more rapidly than its platinum-sensitive counterpart PEO1, while PEO14 and PEO23 have similar proliferation rates.

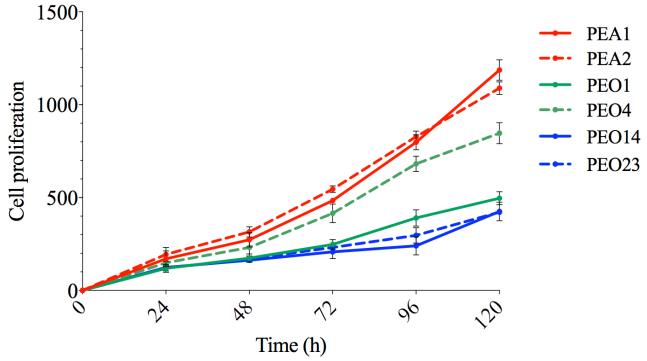


Figure 3.2: Comparison of the growth of the three pairs of HGSOC cell lines
The graph represents the percentage proliferation rate over 120 h for the 6 cell lines. Each point is based on an average of 6 technical replicates and 3 biological replicates ± SEM.

3.2.2 HER-targeted TKI effects on growth

The anti-proliferative effects of the TKI panel were assessed and compared in the three pairs of HGSOC cell lines. All TKIs inhibited the growth of all cell lines under investigation in a concentration-dependent manner, as shown in Figure 3.3. The PEA1-PEA2 cell line pair was the most sensitive pair overall to all TKIs, the PE01-PE04 pair had similar sensitivity, while the least sensitive pair was the PEO14-PEO23 cell line pair. The PE014 cell line, in particular, was markedly more resistant to the inhibitors, than the other cell lines. Neratinib was the most potent inhibitor overall, with IC_{50} values less than 1.05 µM across all cell lines, whereas lapatinib had IC_{50} values ranging from 0.5 to 6 µM, making it the least potent TKI.

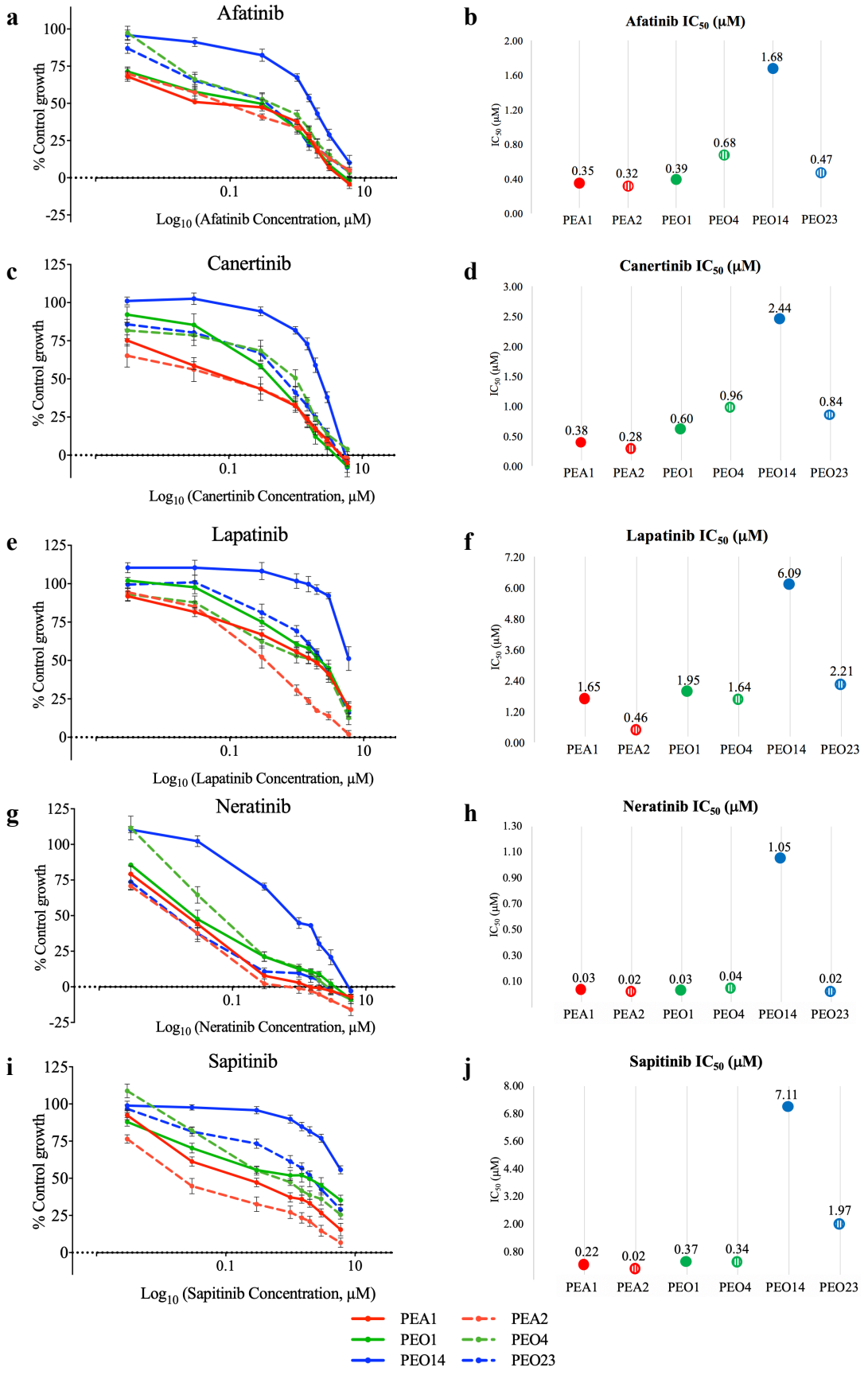


Figure 3.3: Effect of the TKIs on cellular growth of the cell line panel

(a) afatinib concentration-response curves, (b) afatinib IC_{50} values, (c) canertinib concentration-response curves, (d) canertinib IC_{50} values, (e) lapatinib concentration-response curves, (f) lapatinib IC_{50} values, (g) neratinib concentration-response curves, (h) neratinib IC_{50} values, (i) sapitinib concentration-response curves, and (j) sapitinib IC_{50} values. The TKIs at concentrations ranging between 0.003-6 µM were incubated for 120 h. Data are represented as average of 6 technical replicates and least 5 biological replicates ± SEM. The day 0 value is subtracted from the timed value and normalised to the vehicle control.

3.2.3 Pertuzumab and trastuzumab effects on cell line growth

The effects of the HER2-targeted mAbs were investigated after a 120 h incubation with all cell lines to compare with the effects of HER-targeted TKIs. Both mAbs had only modest effects on growth inhibition. The combination treatment of both mAbs did not produce more pronounced results than the single agents.

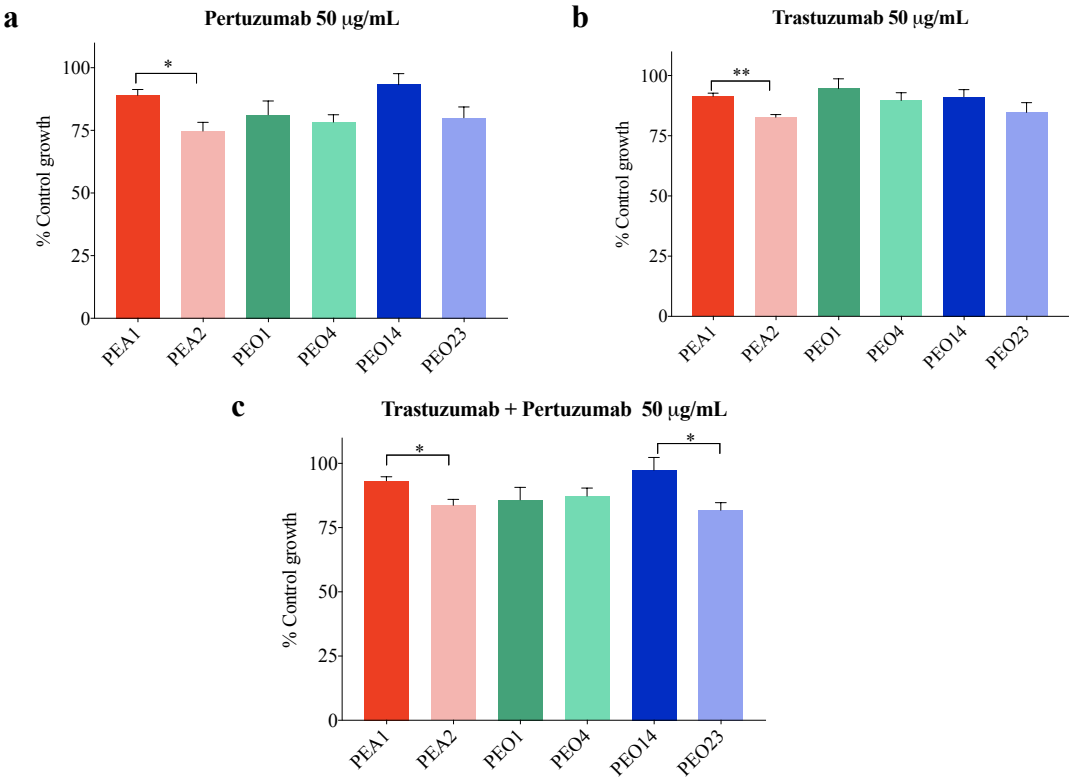


Figure 3.4: Effect of the mAbs on cellular growth of the cell line panel

*Bar graphs shows treatment after 120 h with (a) 50 µg/mL pertuzumab, (b) 50 µg/mL trastuzumab and (c) 25 µg/mL pertuzumab + 25 µg/mL trastuzumab. Data are represented as average of 6 technical replicates and 3 biological replicates ± SEM. The day 0 value is subtracted from the timed value and normalised to the vehicle control. Statistical analysis was performed by unpaired t-test (where *P≤0.05, and **P≤0.01).*

3.2.4 Cytotoxic agent effects on cell growth

Cisplatin has been the standard treatment in patients presenting with ovarian cancer in the past before being superseded by carboplatin. Response to the drug served as an indicator of prognosis, whereby patients who did not respond initially to this agent generally had an overall poor prognosis [356]. The cell lines were derived from patients who were exposed to cisplatin therapy and had become resistant to this platinum-agent. Hence, an SRB proliferation assay was conducted to confirm the cisplatin sensitivity and resistance in the cell lines being investigated. The platinum-sensitive counterparts were, as expected, more sensitive to this agent than their resistant counterparts, with 2.1-fold, 3.5-fold and 2.5-fold difference in IC_{50} concentrations between the PEA1-PEA2, PEO1-PEO4 and PEO14-PEO23 cell line pairs respectively (Figure 3.5 a, b).

As carboplatin would be the current treatment of choice for patients with ovarian cancer, its effects were investigated to compare with those of cisplatin. The cisplatin-sensitive cell lines were more sensitive than the cisplatin-resistant cell lines to carboplatin, but not to the same extent as for cisplatin treatment. Specifically, PEA1-PEA2 pair had a 1.2-fold difference, PEO1-PEO4 cell lines had a 3.4-fold difference, while the PEO14-PEO23 pair had a 1.4-fold change (Figure 3.5 c, d).

The taxane, paclitaxel, was also investigated for its cytotoxic effects on the three cell line pairs as it is often combined with platinum-based agents for the treatment of ovarian cancer. In contrast to results with the carboplatin and cisplatin- treated PEO1-PEO4 cell line pair, paclitaxel caused only a 0.9-fold change between cisplatin-sensitive and resistance cell lines, whereas with platinum treatment, this pair had a more than 3.5-fold variation (Figure 3.5 e, f). The PEA1-PEA2 cell line pair had the greatest fold change, with a 2.3-fold difference between the platinum-sensitive and resistant lines, compared with the <2-fold change in platinum treatments.

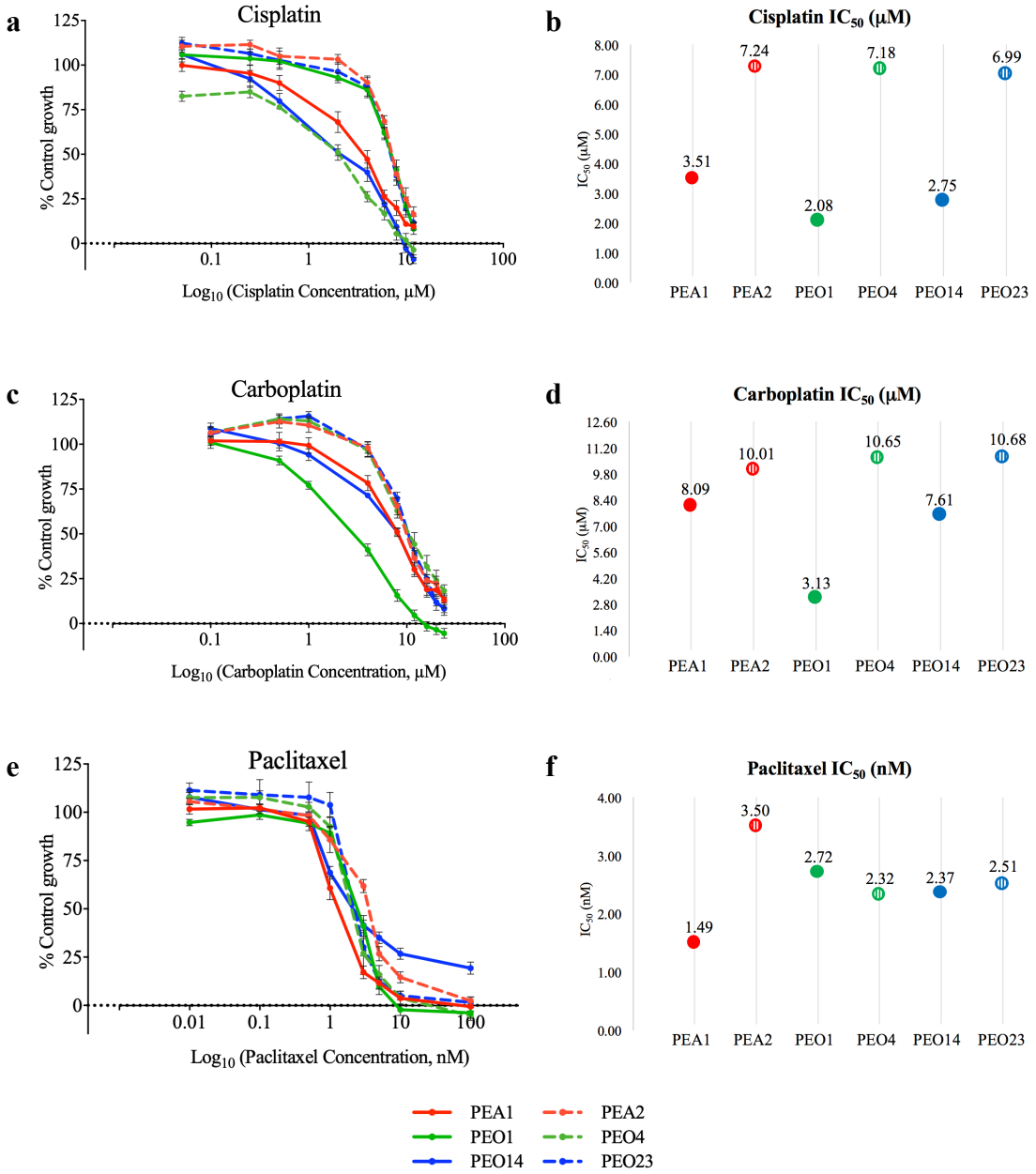


Figure 3.5: Effect of cytotoxic agents on cellular growth of the cell line panel

(a) *cisplatin concentration-response curves, (b) cisplatin IC_{50} values, (c) carboplatin concentration-response curves, (d) carboplatin IC_{50} values, (e) paclitaxel concentration-response curves, and (f) paclitaxel IC_{50} values. The cytotoxic agents were incubated for 120 h and concentrations ranged between 0.05-12 µM for cisplatin, 0.1-24 µM for carboplatin and 0.01-100 nM for paclitaxel. Data are represented as average of 6 technical replicates and at least 5 biological replicates ± SEM. The day 0 value is subtracted from the timed value and normalised to the vehicle control.*

3.3 Effects of combinatorial agents on cell growth

The response of a series of two-drug combinations on the growth of ovarian cancer cell lines was next investigated, using the PEA1 and PEA2 cell line pair. The cytotoxic agents carboplatin, cisplatin and paclitaxel were used at a constant concentration based on their IC_{20} and IC_{30} in each cell line. Trastuzumab and pertuzumab were used at 25 μg/mL or 50 μg/mL. Each cytotoxic agent and mAb was combined with lapatinib or neratinib. These TKIs were chosen as lapatinib is more established and is a widely used TKI in cancer treatment, whereas neratinib has been recently approved in breast cancer treatment and shows good clinical potential. Additionally, lapatinib is a reversible TKI, whilst neratinib is the most potent TKI and has irreversible characteristics. The concentrations of these agents tested were calculated based on eight times their IC_{50}, followed by up to eight doubling dilutions. The two agents were either administered simultaneously or the TKIs were administered for 2 h before the cytotoxic agents or mAbs were added. The cell lines were pre-exposed for a short period because the TKIs have been shown to work within a short period in these cell lines. One rationale behind pre-exposing the cells to the TKIs was because previous studies suggest that the HER pathway is involved with the DNA-repair process [343]. In fact, previous studies whereby ovarian cancer cell lines were treated with a MAPK inhibitor 30 min before the addition of cisplatin exhibited better anti-proliferative effects [357]. The CI method would indicate whether the addition of another drug would sensitise the cell lines to the cytotoxic agents and mAbs. The CI was calculated using CompuSyn Software as explained in section 2.2.1.1.

Cisplatin or carboplatin (± paclitaxel) are first line treatment for ovarian cancer patients. Although HGSOC patients are normally sensitive to this combination, some patients do not achieve the full benefit from monotherapy while others become resistant to this platinum-based agent. The combination of the cytotoxic agents and the TKIs was sought to evaluate whether the TKIs would be able to sensitize the cell lines. Some combinations were previously assessed *in vivo* using lapatinib, but not neratinib, and outcomes were promising [358]. HER-targeted mAbs are not currently used in ovarian cancer patients and few combination investigations with HER-targeted TKIs

have been carried out. However, breast cancer patients who were administered trastuzumab in combination with lapatinib have achieved favourable outcomes [359].

3.3.1 TKIs + cisplatin

The combination of multiple concentrations of lapatinib with cisplatin in PEA1 and PEA2 cell lines mostly produced additive effects, as most CI values ranged from 0.9-1.3 (Figure 3.6 a, b). On the other hand, pre-treatment with neratinib produced synergistic effects in PEA1 and PEA2 cell lines at selected concentrations (Figure 3.6 c-e), especially with the higher cisplatin concentrations. Simultaneous administration of neratinib with cisplatin mostly produced additive effects.

a

PEA1		Lapatinib µM	Sim.	Pre.
[Cisplatin] µM	1.6	0.24	0.90	0.87
		0.48	1.23	1.03
		0.95	1.32	1.37
		1.90	1.91	1.63
		3.80	2.36	2.41
		7.60	1.77	2.51
[Cisplatin] µM	2.43	0.24	0.95	0.95
		0.48	1.02	0.92
		0.95	1.13	1.17
		1.90	1.46	1.29
		3.80	2.28	1.90
		7.60	2.50	2.29

b

PEA2		Lapatinib µM	Sim.	Pre.
[Cisplatin] µM	4.9	0.03	4.73	1.39
		0.06	3.98	1.28
		0.13	2.05	1.46
		0.25	1.96	1.58
		0.51	2.05	1.73
		1.01	2.05	1.79
[Cisplatin] µM	5.6	0.03	1.12	1.89
		0.06	1.29	2.19
		0.13	1.12	1.97
		0.25	0.96	2.22
		0.51	0.84	1.83
		1.01	1.31	1.65

c

PEA1		Neratinib µM	Sim.	Pre.
[Cisplatin] µM	1.6	0.003	1.11	0.75
		0.006	0.99	0.32
		0.013	1.20	0.51
		0.026	1.21	0.52
		0.051	1.30	0.65
		0.103	1.25	0.78
[Cisplatin] µM	2.43	0.003	1.14	0.29
		0.006	1.16	0.31
		0.013	1.31	0.32
		0.026	1.23	0.36
		0.051	1.28	0.45
		0.103	1.40	0.57

d

PEA2		Neratinib µM	Sim.	Pre.
[Cisplatin] µM	4.9	0.0006	1.19	3.42
		0.0012	1.16	1.61
		0.0023	1.32	0.84
		0.0046	1.67	0.82
		0.0093	1.83	0.74
		0.0185	2.15	0.99
[Cisplatin] µM	5.6	0.0006	1.25	0.78
		0.0012	1.32	0.59
		0.0023	1.43	0.48
		0.0046	1.77	0.47
		0.0093	2.07	0.44
		0.0185	2.59	0.51

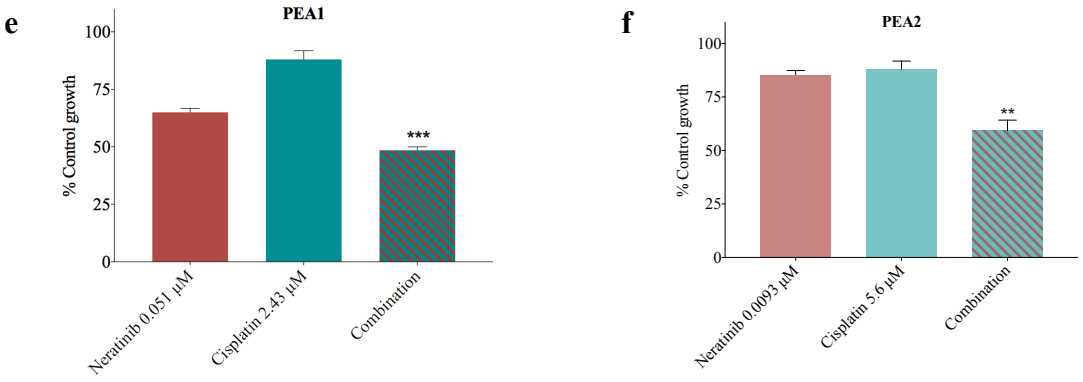
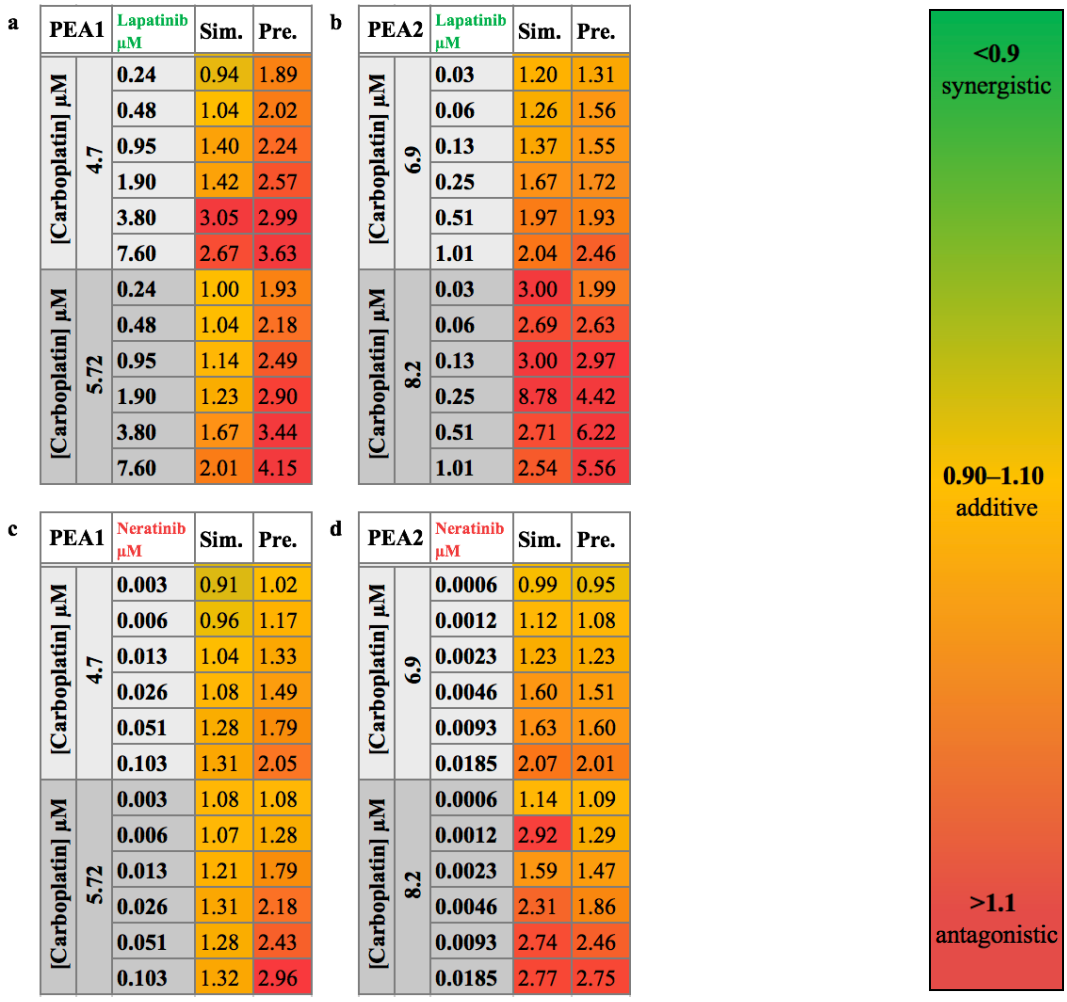


Figure 3.6: Combination index for TKIs + cisplatin

*(a) CI values for lapatinib + cisplatin in PEA1, (b) CI values for lapatinib + cisplatin in PEA2, (c) CI values for neratinib + cisplatin in PEA1 (d) CI values for neratinib + cisplatin in PEA2. The CI values were calculated using CompuSyn software. The data used were a mean of 3 technical replicates and 3 biological replicates. The abbreviations "Sim." and "Pre." refer to simultaneous treatment and pre-treatment with TKIs respectively. (e) bar graph of inhibitory effect in PEA1 with neratinib 0.051 µM + cisplatin 2.43 µM combination, (f) bar graph of inhibitory effect in PEA2 with neratinib 0.0093 µM + cisplatin 5.6 µM combination. The bar graphs represent mean ± SEM, and statistical analysis was conducted using two-way ANOVA (where **P≤0.01, *** P≤0.001).*

3.3.2 TKIs + carboplatin

The combination of lapatinib and carboplatin was next compared and this typically resulted in additive or antagonistic effects in PEA1 and PEA2 cell lines, especially when pre-treating cells with lapatinib (Figure 3.7 a, b). Combining neratinib and carboplatin produced predominantly additive effects in PEA1 and PEA2 cell lines (Figure 3.7 c, d).



a

PEA1	Lapatinib µM	Sim.	Pre.
[Carboplatin] µM 4.7	0.24	0.94	1.89
	0.48	1.04	2.02
	0.95	1.40	2.24
	1.90	1.42	2.57
	3.80	3.05	2.99
	7.60	2.67	3.63
[Carboplatin] µM 5.72	0.24	1.00	1.93
	0.48	1.04	2.18
	0.95	1.14	2.49
	1.90	1.23	2.90
	3.80	1.67	3.44
	7.60	2.01	4.15

b

PEA2	Lapatinib µM	Sim.	Pre.
[Carboplatin] µM 6.9	0.03	1.20	1.31
	0.06	1.26	1.56
	0.13	1.37	1.55
	0.25	1.67	1.72
	0.51	1.97	1.93
	1.01	2.04	2.46
[Carboplatin] µM 8.2	0.03	3.00	1.99
	0.06	2.69	2.63
	0.13	3.00	2.97
	0.25	8.78	4.42
	0.51	2.71	6.22
	1.01	2.54	5.56

c

PEA1	Neratinib µM	Sim.	Pre.
[Carboplatin] µM 4.7	0.003	0.91	1.02
	0.006	0.96	1.17
	0.013	1.04	1.33
	0.026	1.08	1.49
	0.051	1.28	1.79
	0.103	1.31	2.05
[Carboplatin] µM 5.72	0.003	1.08	1.08
	0.006	1.07	1.28
	0.013	1.21	1.79
	0.026	1.31	2.18
	0.051	1.28	2.43
	0.103	1.32	2.96

d

PEA2	Neratinib µM	Sim.	Pre.
[Carboplatin] µM 6.9	0.0006	0.99	0.95
	0.0012	1.12	1.08
	0.0023	1.23	1.23
	0.0046	1.60	1.51
	0.0093	1.63	1.60
	0.0185	2.07	2.01
[Carboplatin] µM 8.2	0.0006	1.14	1.09
	0.0012	2.92	1.29
	0.0023	1.59	1.47
	0.0046	2.31	1.86
	0.0093	2.74	2.46
	0.0185	2.77	2.75

Figure 3.7: Combination index for TKIs + carboplatin

(a) CI values for lapatinib + carboplatin in PEA1, (b) CI values for lapatinib + carboplatin in PEA2, (c) CI values for neratinib + carboplatin in PEA1 (d) CI values for neratinib + carboplatin in PEA2. The CI values were calculated using CompuSyn software. The data used were a mean of 3 technical replicates and 3 biological replicates. The abbreviations "Sim." and "Pre." refer to simultaneous treatment and pre-treatment with TKIs respectively.

3.3.3 TKIs + paclitaxel

The combination of lapatinib with paclitaxel produced mainly synergistic effects in PEA1 cells (Figure 3.8 a). On the other hand, lapatinib with paclitaxel had additive effects in PEA2 cells (Figure 3.8 b). When neratinib was combined with paclitaxel, it mainly resulted in additive effects in PEA1 cells. In the PEA2 cell line, pre-treatment with neratinib and the addition of low concentration paclitaxel produced synergistic effects, while the higher concentration of paclitaxel resulted in antagonistic effects (Figure 3.8 c, d).

a

PEA1		Lapatinib µM	Sim.	Pre.
[Paclitaxel] µM	0.001	0.24	0.38	0.34
		0.48	0.33	0.33
		0.95	0.43	0.32
		1.90	0.48	0.31
		3.80	0.60	0.40
		7.60	0.68	0.48
[Paclitaxel] µM	0.0015	0.24	0.20	0.43
		0.48	0.40	0.31
		0.95	0.52	0.45
		1.90	1.22	0.41
		3.80	1.01	0.46
		7.60	0.48	0.45

b

PEA2		Lapatinib µM	Sim.	Pre.
[Paclitaxel] µM	0.002	0.03	0.85	1.00
		0.06	0.88	1.01
		0.13	0.91	1.06
		0.25	1.03	1.13
		0.51	1.02	1.45
		1.01	1.03	1.64
[Paclitaxel] µM	0.003	0.03	1.52	1.02
		0.06	1.44	0.96
		0.13	1.50	1.21
		0.25	1.55	1.73
		0.51	1.65	1.78
		1.01	1.73	2.07

c

PEA1		Neratinib µM	Sim.	Pre.
[Paclitaxel] µM	0.001	0.003	0.88	0.91
		0.006	0.77	0.91
		0.013	0.76	1.01
		0.026	1.19	0.94
		0.051	0.98	0.89
		0.103	1.02	0.98
[Paclitaxel] µM	0.0015	0.003	0.94	0.87
		0.006	1.03	0.91
		0.013	1.08	0.81
		0.026	1.27	0.79
		0.051	0.92	1.02
		0.103	0.89	1.08

d

PEA2		Neratinib µM	Sim.	Pre.
[Paclitaxel] µM	0.002	0.0006	0.78	0.49
		0.0012	0.83	0.51
		0.0023	0.76	0.59
		0.0046	0.94	0.72
		0.0093	0.99	0.83
		0.0185	1.18	0.95
[Paclitaxel] µM	0.003	0.0006	2.19	2.17
		0.0012	2.12	2.26
		0.0023	2.13	2.02
		0.0046	2.23	2.12
		0.0093	2.20	2.42
		0.0185	2.36	2.64

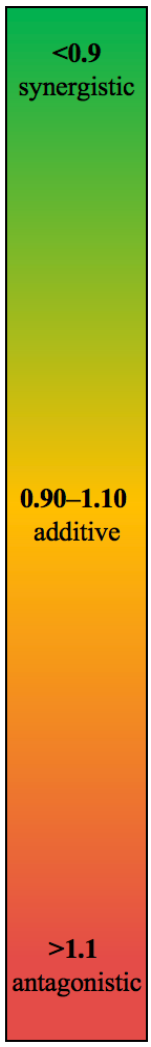


Figure 3.8: Combination index for TKIs + paclitaxel

(a) CI values for lapatinib + paclitaxel in PEA1, (b) CI values for lapatinib + paclitaxel in PEA2, (c) CI values for neratinib + paclitaxel in PEA1 (d) CI values for neratinib + paclitaxel in PEA2. The CI values were calculated using CompuSyn software. The data used were a mean of 3 technical replicates and 3 biological replicates. The abbreviations "Sim." and "Pre." refer to simultaneous treatment and pre-treatment with TKIs respectively.

3.3.4 TKIs + pertuzumab

When pre-treatment of lapatinib or neratinib was combined with pertuzumab, it generally resulted in synergistic effects in the PEA1 cell line. The simultaneous administration of the TKI and mAb had an additive effect (Figure 3.9 a, c). Similarly, the simultaneous administration of lapatinib or neratinib and pertuzumab in PEA2 cell line produced mainly additive effects. However, the pre-treatment of neratinib or lapatinib with pertuzumab had antagonistic outcomes in PEA2 cell line (Figure 3.9 b, d).

a

PEA1		Lapatinib µM	Sim.	Pre.
[Pert.] µg/mL	25	0.24	0.87	0.60
		0.48	0.86	0.47
		0.95	0.87	1.17
		1.90	1.13	1.05
		3.80	1.81	1.53
		7.60	1.34	1.65
[Pert.] µg/mL	50	0.24	0.98	0.30
		0.48	1.22	0.38
		0.95	1.31	0.54
		1.90	1.39	0.78
		3.80	1.61	1.22
		7.60	1.80	1.31

b

PEA2		Lapatinib µM	Sim.	Pre.
[Pert.] µg/mL	25	0.03	0.96	1.68
		0.06	0.79	1.59
		0.13	0.79	2.50
		0.25	1.01	4.50
		0.51	1.04	5.00
		1.01	1.57	6.00
[Pert.] µg/mL	50	0.03	0.72	3.50
		0.06	0.46	5.50
		0.13	0.57	6.20
		0.25	0.50	6.56
		0.51	1.86	6.90
		1.01	1.01	7.00

c

PEA1		Neratinib µM	Sim.	Pre.
[Pert.] µg/mL	25	0.003	0.43	0.60
		0.006	0.80	0.70
		0.013	1.04	0.76
		0.026	1.40	0.86
		0.051	0.84	0.77
		0.103	0.92	0.93
[Pert.] µg/mL	50	0.003	0.74	0.61
		0.006	1.03	0.91
		0.013	1.06	0.84
		0.026	0.94	1.07
		0.051	0.85	0.86
		0.103	1.07	1.08

d

PEA2		Neratinib µM	Sim.	Pre.
[Pert.] µg/mL	25	0.0006	0.89	0.21
		0.0012	1.61	0.62
		0.0023	0.33	0.62
		0.0046	0.37	3.07
		0.0093	0.61	3.91
		0.0185	0.91	7.78
[Pert.] µg/mL	50	0.0006	0.39	3.79
		0.0012	0.42	3.83
		0.0023	0.43	2.50
		0.0046	0.39	8.00
		0.0093	0.50	9.00
		0.0185	0.65	11.00

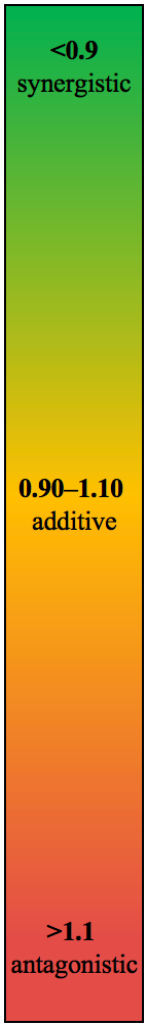


Figure 3.9: Combination index for TKIs + pertuzumab

(a) CI values for lapatinib + pertuzumab in PEA1, (b) CI values for lapatinib + pertuzumab in PEA2, (c) CI values for neratinib + pertuzumab in PEA1 (d) CI values for neratinib + pertuzumab in PEA2. The CI values were calculated using CompuSyn software. The data used were a mean of 3 technical replicates and 3 biological replicates. The abbreviations "Sim." and "Pre." refer to simultaneous treatment and pre-treatment with TKIs respectively, while "Pert." refers to pertuzumab.

3.3.5 TKIs + trastuzumab

The combination of trastuzumab with lapatinib or neratinib mainly produced additive or antagonistic effects. In PEA2 cells, the combination generally resulted in antagonistic results, especially for pre-treatment with TKIs. On the other hand, simultaneous treatment of the TKIs and trastuzumab produced additive effects in PEA1 (Figure 3.10).

a

PEA1		Lapatinib µM	Sim.	Pre.
[Trast.] µg/mL	25	0.24	1.04	3.02
		0.48	1.18	1.79
		0.95	1.39	2.07
		1.90	1.69	1.59
		3.80	2.38	2.24
		7.60	1.97	1.97
[Trast.] µg/mL	50	0.24	1.99	2.31
		0.48	2.27	2.13
		0.95	2.54	2.04
		1.90	2.37	1.89
		3.80	2.56	2.56
		7.60	3.52	2.68

b

PEA2		Lapatinib µM	Sim.	Pre.
[Trast.] µg/mL	25	0.03	2.61	1.42
		0.06	2.22	1.80
		0.13	1.34	2.35
		0.25	0.77	2.88
		0.51	0.94	3.75
		1.01	1.05	4.75
[Trast.] µg/mL	50	0.03	1.55	3.29
		0.06	0.44	3.33
		0.13	0.72	3.10
		0.25	0.86	3.20
		0.51	1.11	3.50
		1.01	1.31	4.50

c

PEA1		Neratinib µM	Sim.	Pre.
[Trast.] µg/mL	25	0.003	1.03	2.93
		0.006	1.54	2.45
		0.013	1.75	2.64
		0.026	1.82	2.16
		0.051	1.31	2.11
		0.103	1.24	0.92
[Trast.] µg/mL	50	0.003	1.10	2.93
		0.006	1.02	2.45
		0.013	1.22	2.64
		0.026	1.26	2.16
		0.051	1.29	2.11
		0.103	1.36	0.88

d

PEA2		Neratinib µM	Sim.	Pre.
[Trast.] µg/mL	25	0.0006	1.62	1.07
		0.0012	2.74	1.19
		0.0023	1.70	1.35
		0.0046	1.65	1.53
		0.0093	2.22	1.77
		0.0185	2.69	2.07
[Trast.] µg/mL	50	0.0006	1.82	1.78
		0.0012	2.01	3.24
		0.0023	2.26	2.29
		0.0046	2.66	2.75
		0.0093	3.05	3.11
		0.0185	3.67	3.83

Figure 3.10: Combination index for TKIs + trastuzumab

(a) CI values for lapatinib + trastuzumab in PEA1, (b) CI values for lapatinib + trastuzumab in PEA2, (c) CI values for neratinib + trastuzumab in PEA1 (d) CI values for neratinib + trastuzumab in PEA2. The CI values were calculated using CompuSyn software. The data used were a mean of 3 technical replicates and 3 biological replicates. The abbreviations "Sim." and "Pre." refer to simultaneous treatment and pre-treatment with TKIs respectively, while "Trast." refers to trastuzumab.

3.4 Effects on cellular migration

To assess the migratory ability of the cell lines under investigation, the scratch assay was undertaken, whereby a scratch was made across a cellular monolayer and the percentage migration into the created gap at specific time points was measured. The medium in the cells was changed to 0.1% FBS containing medium to limit the effects of proliferation. The cell lines under investigation had differing migratory rates as indicated in Figure 3.11. The PEA1-PEA2 pair of cell lines were the most migratory, migrating over 35% in 24 h. On the other hand, the least migratory pair was the PEO1-PEO4 pair, which only migrated about 14% in 24 h.

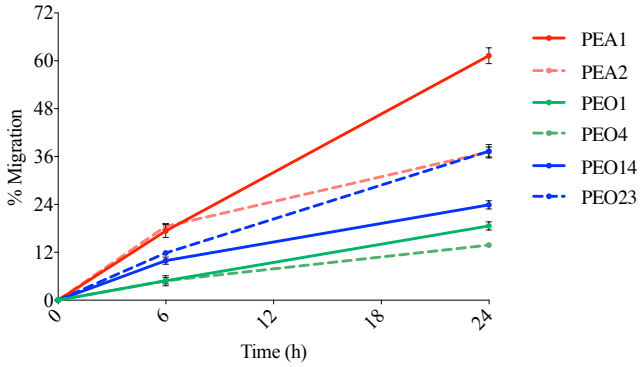


Figure 3.11: Migration of the panel of cell lines over 24 h

The graph shows the migration properties of all cell lines in a 24 h period, with no added inhibitors or growth factors. Data are represented as average of 6 technical replicates and at least 4 biological replicates ± SEM. The data are normalised to 0 h.

3.4.1 Effect of TKIs on migration

Migration may be driven by downstream pathways that are shared with HER pathways [360]. The abilities of the five TKIs to inhibit migration in the panel of cell lines was next evaluated. The TKIs were added to the wells at concentrations which are achievable in patient sera at the maximum tolerated dose of treatment [307, 310, 312, 314, 361], thus in accordance with the concentrations potentially available within *in vivo* tumours.

The TKIs reduced migration to different extents, as can be seen in Figure 3.12. In the PEA1-PEA2 pair of cell lines, the most effective TKI was sapitinib, which inhibited migration by 56% and 68% respectively (Table 3.1). On the other hand, lapatinib was

the least effective inhibitor especially in the PEA1 cell line, where it only inhibited migration by 18%. In general, the PEA2 cell line was more sensitive to the anti-migratory properties of the TKIs.

For the PEO1 cell line, neratinib was the most effective TKI, while sapitinib produced better inhibitory outcomes in the PEO4 cell line. The least effective TKI effects were with afatinib in the PEO1 and neratinib in the PEO4 cell lines. Similar to the PEA1-PEA2 cell line pair, the platinum-resistant counterpart was more sensitive to the TKI anti-migratory effects.

Comparable to the PEA1-PEA2 cell line pair, sapitinib was the most effective inhibitor in the PEO14-PEO23 cell line pair inhibiting migration by 70% and 28% respectively. The least potent inhibitors were lapatinib in the PEO14 and canertinib in the PEO23 cell lines.

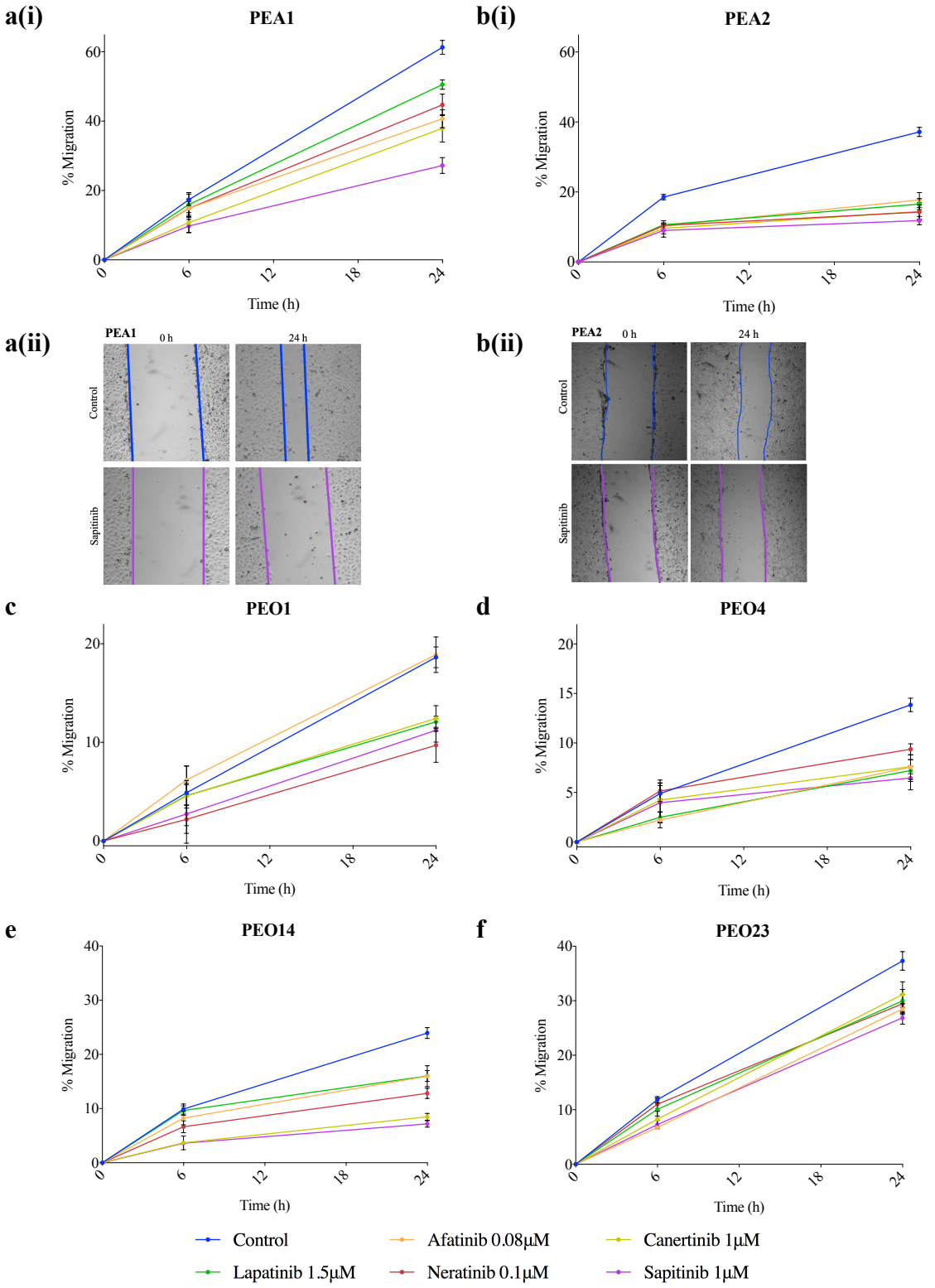


Figure 3.12: TKIs inhibit migration of all cell lines

The graphs show the migration properties of all cell lines in a 24 h period, when TKIs were added. (a) PEA1 cell line, (b) PEA2 cell line, (c) PEO1 cell line, (d) PEO4 cell line, (e) PEO14 cell line and (f) PEO23 cell line. Data are represented as average of 6 technical replicates and at least 4 biological replicates ± SEM. The data are normalised to 0 h. Statistical analysis was performed by two-way ANOVA.

	PEA1	PEA2	PEO1	PEO4	PEO14	PEO23
Afatinib (0.08 µM)	34%	52%	-1%	45%	33%	24%
Canertinib (1 µM)	38%	61%	33%	45%	65%	17%
Lapatinib (1.5 µM)	18%	56%	35%	48%	33%	20%
Neratinib (0.1 µM)	27%	62%	48%	32%	47%	21%
Sapitinib (1 µM)	56%	68%	40%	53%	70%	28%

Table 3.1: Percentage relative inhibition on migration compared to control at 24 h

*Calculation: ((a-b)/a)*100; where a=% migrated by control, b= % migrated by TKI, as explained in section 2.2.2. Migration inhibition greater than 35% (median migration inhibition) is shown in green, whilst migration inhibition less than 35% is shown in red.*

3.4.2 Effect of mAbs on migration

The PEA1-PEA2 pair of cell lines was chosen to assess the inhibitory role of HER2-targeted mAbs on ovarian cell line migration, as these cell lines were the most migratory of the panel investigated. The platinum-resistant cell line, PEA2, was markedly more sensitive to the mAb effect on migration than its PEA1 partner, which expressed less HER2 than PEA2 cell line. The combination of pertuzumab and trastuzumab (25+25 µg/mL) had the greatest effect on migration in both cell lines compared to monotherapy, especially in PEA2 cells with almost 58% migration inhibition, as seen in Figure 3.13 and presented in Table 3.2. As monotherapy, trastuzumab was more effective than pertuzumab in the PEA2 cell line.

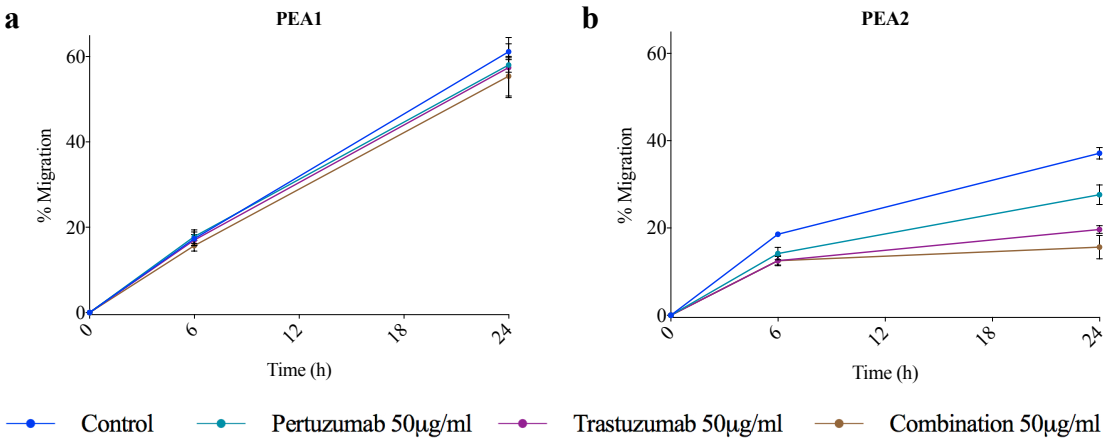


Figure 3.13: mAbs inhibit PEA2 cell line migration

The graphs show the migration properties of PEA1-PEA2 cell lines in a 24 h period, when mAbs were added. (a) PEA1 cell line, (b) PEA2 cell line. Data are represented as average of 6 technical replicates and 3 biological replicates ± SEM. The data are normalised to 0 h.

	PEA1	PEA2
Pertuzumab (50 µg/mL)	5%	26%
Trastuzumab (50 µg/mL)	6%	47%
Combination (25+25 µg/mL)	9%	58%

Table 3.2: Percentage relative inhibition on migration compared to control at 24 h
*Calculation: ((a-b)/a)*100; where a=% migrated by control, b= % migrated by mAb.*

3.5 Effects on cellular adhesion

The adherence properties of the cell lines to different ECMs was next investigated (Figure 3.14). The 96-well plates were coated with either collagen, fibronectin or laminin and the cells were seeded into the coated or non-coated plates and left to incubate for 1 h before they were fixed. It is evident that the cells were able to adhere more to collagen, and least to laminin. The platinum-resistant cell lines adhered more to collagen and fibronectin than their corresponding platinum-sensitive cell line.

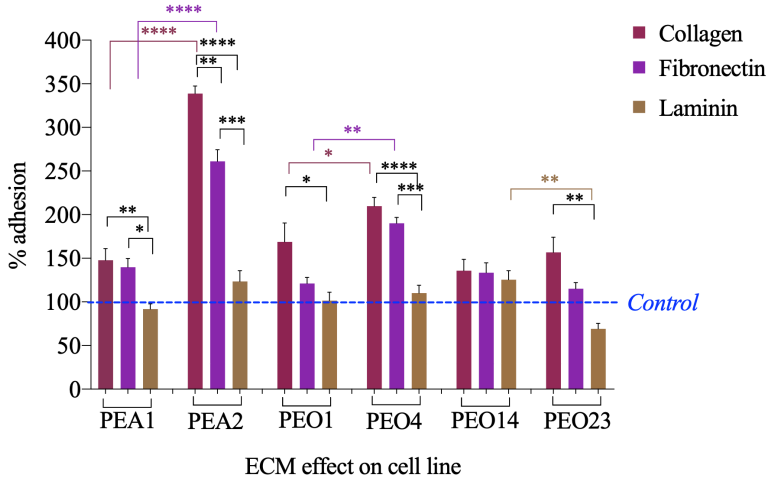


Figure 3.14: Cell line adherence to ECM components
*The graph indicates the extent of cell line adherence to each ECM on the 6 cell lines after 1 h. The values show the percentage of cells that adhered to each of the ECM-coated wells normalised to the cells adhered to the non-coated wells, seeded in normal media. Data are presented as mean of 3 technical replicates and 5 biological replicates ± SEM. Statistical analysis was performed by unpaired t-test between each cell line pair within specific ECMs, and by one-way ANOVA to analyse the statistical significance of the different ECMs within each cell line (where *P≤0.05, **P≤0.01, *** P≤0.001, and ****P<0.0001).*

Neratinib was chosen to test the effect of TKIs on adhesion, as it had good inhibitory effects on growth and migration at or less than its therapeutic concentration. Additionally, neratinib is a novel, pan-HER agent. Neratinib was able to reduce

adhesion in all fibronectin-exposed cell lines, but not as well in the PEA1 and PEA2 cell lines (Figure 3.15). However, it did not inhibit the adhesion of collagen- and laminin- exposed PEO14 cells. The inhibition was greater in the PEO1-PEO4 cell line pair. In the PEA1-PEA2 and PEO14-PEO23 cell line pairs, neratinib had a more potent effect on the platinum-resistant cell lines.

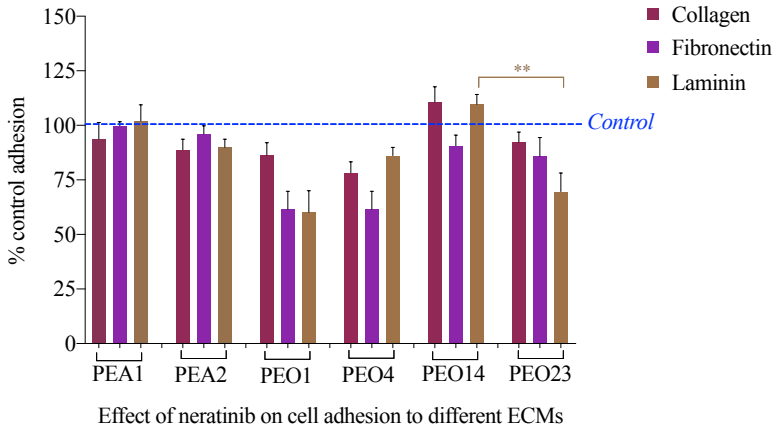


Figure 3.15: Neratinib reduces adhesion after 1 h

*The graph represents the effect of neratinib on adhesion of cells to each ECM after 1 h. The values shown were calculated as follows: ((a-b)/(c-d))*100; where a= ECM control, b= uncoated control, c= ECM + neratinib and d= neratinib control. Data are presented as mean of 3 technical replicates and 5 biological replicates ± SEM. Statistical analysis was performed by unpaired t-test between each cell line pair within specific ECMs, and by one-way ANOVA to analyse the statistical significance of the different ECMs within each cell line (where **P≤0.01).*

3.6 Effects on invasion

Invasion is one of the defining hallmarks of cancer [5]. To investigate this, cell lines were grown in spinner flasks to form 3D spheroids. All cell lines, except PEA1, formed spheroids in sufficient sizes that could be used for the invasion assay. Spheroids were implanted into a collagen matrix and invasion was monitored by microscopy over a 96 h period. Percentage invasion was calculated by normalising the area at each time point and comparing this with the area at 0 h.

The relative invasion capacity of all cell lines is presented in Figure 3.16. The PEO1-PEO4 pair of cell lines invaded more than the other cell lines, with over 6-fold increase compared to its original size. The PEO23 cell line was the least invasive.

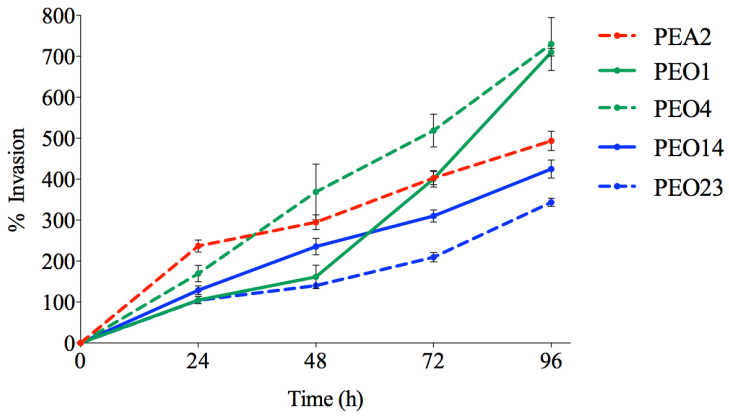


Figure 3.16: Comparison of the invasion properties across the three HGSOC cell lines

The graph shows the invasion properties of all cell lines over a 96 h period, in collagen mix with no added inhibitors or growth factors. Data are represented as average of 4 technical replicates and at least 2 biological replicates ± SEM. The data are normalised to 0 h.

3.6.1 Neratinib effects on invasion

Spheroids were also treated with neratinib at a concentration of 0.1 μM to investigate its anti-invasive action. Neratinib was again chosen as the representative TKI due to the novelty of assessing its use in *in vitro* invasion assays.

Neratinib reduced invasion in ovarian cancer cell lines, as illustrated in Figure 3.17. It reduced invasion by over 70% in PEO14, and by almost half in the PEA2 cell line (Table 3.3).

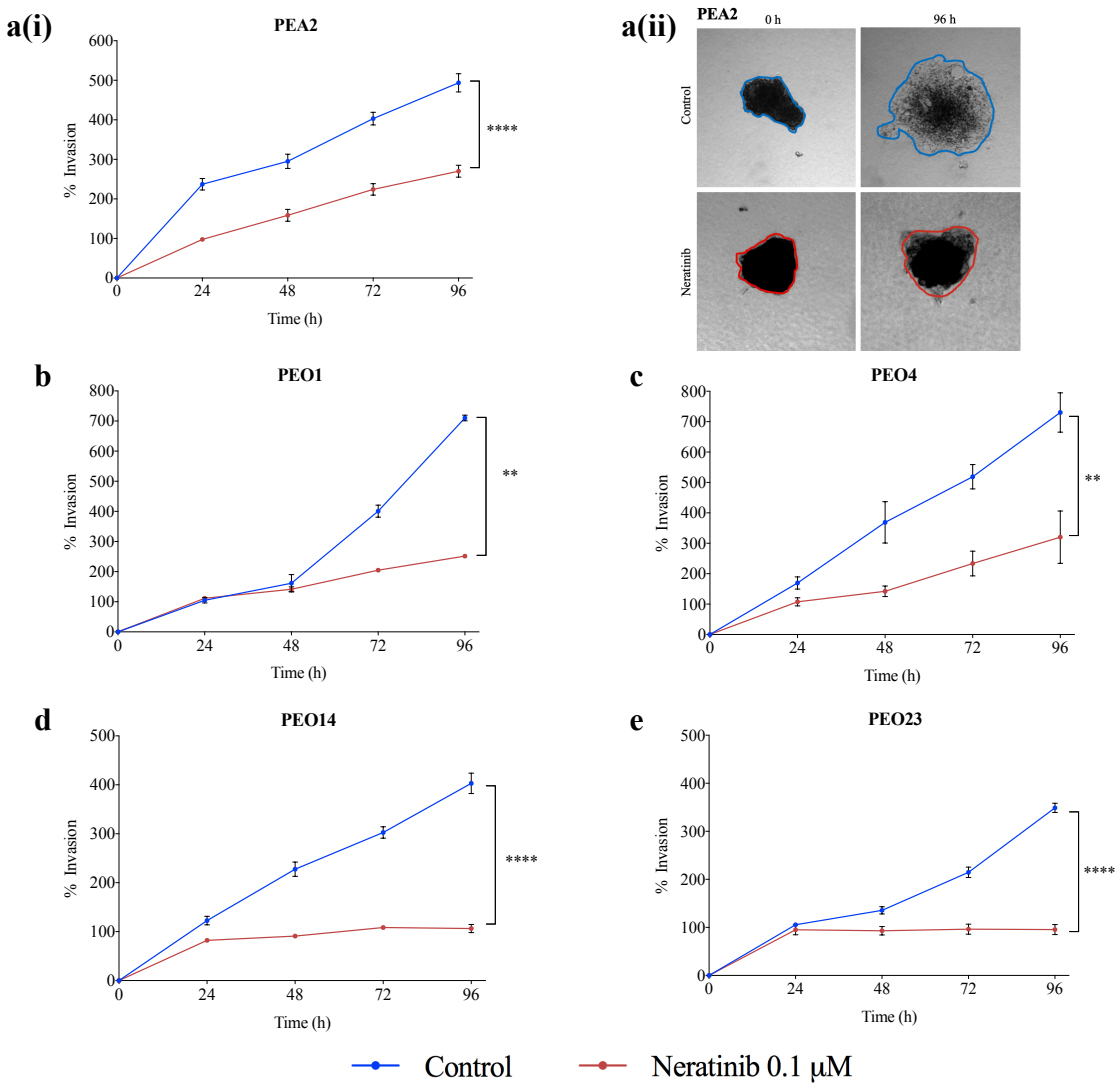


Figure 3.17: Neratinib inhibits invasion of the cell lines

*The graphs represent the effects of neratinib on invasion in (a) PEA2, (b) PEO1, (c) PEO4, (d) PEO14 and (e) PEO23. Each point is normalised to 0 h and data represent mean of 4 technical replicate and at least 2 biological replicates. Statistical analyses were performed using two-way ANOVA (where **P≤0.01, and ****P<0.0001).*

	PEA2	PEO1	PEO4	PEO14	PEO23
Neratinib	45%	65%	56%	71%	71%

Table 3.3: Percentage inhibition on invasion relative to control at 96 h

*Calculation: ((a-b)/a)*100; where a=% invaded by control, b= % invaded by neratinib, as explained in section 2.2.4.*

3.7 Discussion

3.7.1 TKIs inhibit growth in HGSOC cell lines

One of the main aims of this study was to investigate the effect of different HER-targeted therapies on the growth of platinum-resistant and platinum-sensitive HGSOC cell line pairs. To do this, HER-targeted TKIs and mAbs were compared. The cell lines under investigation had different levels of expressions of the HER receptors, which allowed determination of whether a particular profile of HER expression conferred more sensitivity than another. Generally, higher expression of HER3 was associated with a lower proliferation rate.

All the TKIs investigated in this study caused a reduction in cellular proliferation, in a concentration-dependent manner. The most potent TKI amongst the ones studied was neratinib, as tabulated in Table 3.4, which targets EGFR, HER2 and HER4 receptors [298]. The IC_{50} values in PEA2 were all under the serum concentrations achieved *in vivo* after maximum tolerated dose administration [307, 312, 314, 361], except for afatinib, which was considerably higher, taking into account its serum concentration *in vivo* is about 0.1 μM [310]. The most sensitive cell line pair was the PEA1-PEA2 cell line pair, while the least sensitive pair was the PEO14-PEO23 cell line pair. Interestingly, the PEA1-PEA2 cell lines had the greatest proliferation rates, while PEO14-PEO23 had the slowest. The PE014 cell line was most resistant to the TKI inhibitors and also to Pertuzumab and the antibody combination. This may be associated with the lower levels of EGFR and HER2 expression found in this cell line relative to the other cell lines leading to a reduced level of dependency on these receptors.

The reversible EGFR- and HER2-targeting TKI lapatinib [251] was the least potent agent. Lapatinib had the highest IC_{50} values amongst all the TKIs in all the cell lines. The IC_{50} values are higher than the therapeutic concentration usually achieved by lapatinib *in vivo* [361], except in PEA2. Thus, the cell lines were generally resistant to lapatinib. The PEO14 and PEO23 cell lines were both resistant to sapitinib therapy as their IC_{50} values were higher than the clinically relevant concentration [307]. The

platinum-resistant cell lines PEA2 and PEO23 were more sensitive to the TKI therapy than their platinum-sensitive counterpart. With regard to the other platinum-resistant cell line, PEO4, this only responded better than PEO1 to sapitinib and lapatinib treatment. Although canertinib usually acts more effectively in the presence of low HERs [285], it was not the case in these cell lines, as the most resistant cell line was PEO14 cell line, which did not express high levels of HERs.

	Increased potency				
Increased sensitivity ↑					
PEA2	Neratinib	Sapitinib	Canertinib	Afatinib	Lapatinib
PEA1	Neratinib	Sapitinib	Afatinib	Canertinib	Lapatinib
PEO1	Neratinib	Sapitinib	Afatinib	Canertinib	Lapatinib
PEO4	Neratinib	Sapitinib	Afatinib	Canertinib	Lapatinib
PEO23	Neratinib	Afatinib	Canertinib	Sapitinib	Lapatinib
PEO14	Neratinib	Afatinib	Canertinib	Sapitinib	Lapatinib

Table 3.4: Compilation of IC_{50} values ordered in accordance to potency of TKI and sensitivity of cell line to TKIs

There were no statistically significant correlations between the level of HER expression and the potency of each TKI, which was also noted in other studies [272, 362]. However, there is an increased tendency that higher levels of the HERs, increase TKI potency. With regards to HER3, the relationship mostly existed with sapitinib, which relates to the fact that sapitinib targets HER3.

The two mAbs investigated in this project were not capable of reducing growth under 70% of the control value, which could be because the ovarian cancer cell lines used in this study did not overexpress HER2. Pertuzumab had better inhibitory effects than the combination of pertuzumab and trastuzumab. The concentration used (50 µg/mL) for both mAbs was based on the clinical target concentration to saturate the HER2 receptor (20 µg/mL [363]), and the usual serum concentration achieved by these mAbs (pertuzumab ~70 µg/mL [227, 364], trastuzumab 35-148 µg/mL [365]). Increased EGFR expression was associated with reduced potency of trastuzumab. This is similar to previous breast cancer studies, which concluded that overexpression of EGFR in high HER2 cells was related to trastuzumab resistance [366]. There was a statistically

significant correlation between the HER2 expression and the cell line response to pertuzumab (P<0.05; Spearman rank test), as seen in Figure 3.18.

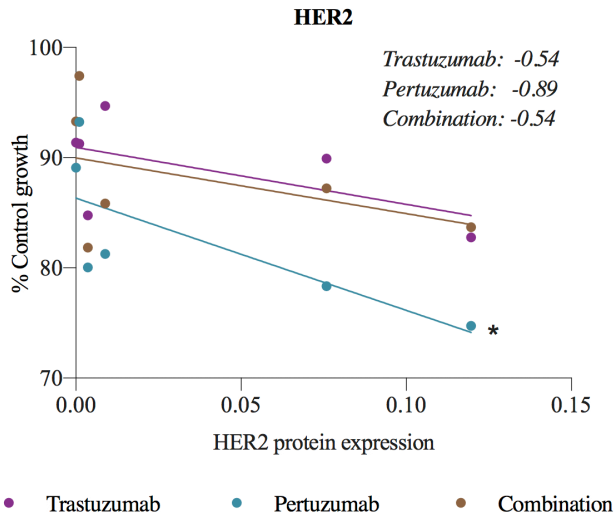


Figure 3.18: Correlation between HER2 protein expression and anti-proliferative response to mAbs

*The results were correlated using Spearman correlation method and the Spearman r values are shown in the graph (where *P≤0.05).*

Three cytotoxic agents (cisplatin, carboplatin and paclitaxel) were also investigated for their effects on proliferation. All three cytotoxic agents were able to decrease the growth of the cell lines, however, the platinum-sensitive cell lines were more sensitive to all three cytotoxic agents. The cisplatin-sensitive cell lines were still sensitive to the agent as their IC_{50} were normally below the usual cisplatin average serum concentration of about 3.3 μM [367, 368]. On the other hand, all the cell lines had IC_{50}s below the usual plasma concentrations of paclitaxel (>5 μM [369, 370]). This suggests that there is no cross-resistance between cisplatin and paclitaxel, which was also noticed *in vivo* [337, 371]. There was no association between the sensitivity to the cytotoxic agents and sensitivity to TKIs or HER expression. However, there seems to be an inverse correlation between the sensitivity to trastuzumab and sensitivity to cisplatin. Previous studies in breast cancer also failed to correlate HER2 expression with response to the cytotoxic agents [372-375], while others attribute high expression of HER2 with reduced response to paclitaxel in breast cancer patients [376].

3.7.2 Combining neratinib with cisplatin and pertuzumab potentiates cytotoxicity

For optimal patient treatment, drugs are often combined as combination treatment can offer several advantages. These advantages include use of lower drug doses as a lower drug dose is frequently still effective which leads to lesser adverse effects and better tolerability. The targeting of multiple cell signalling pathways also reduces the chances of developing drug resistance and can increase sensitivity. However, not all drug combinations are effective, as some drugs might interact with each other and cause antagonistic effects, thus reducing their actual anti-proliferative effect. In this section, the drug combinations that produced additive effects, or better still, synergistic effects are discussed.

Previous studies show that EGFR activation due to cisplatin treatment, reduces the efficacy of cisplatin [184, 185]. Additionally, matuzumab, an anti-EGFR mAb inhibited cisplatin-induced EGFR activation, and resulted in reduced tumour growth [377]. Hence, this study looked at the effects of combining cisplatin with an EGFR inhibitor. The combination of cisplatin and lapatinib tested resulted in additive or antagonistic effects in the two ovarian cancer cell lines in this study. This contradicts other reports demonstrating synergistic results obtained from the cisplatin and lapatinib combination in gastric cancer cell lines [378]. However, the pre-treatment of neratinib with cisplatin produced synergistic effects in the ovarian cancer cell lines. One possible explanation as to why prior TKI administration had synergistic effects but not with simultaneous administration could be that HER receptors may have a role in DNA-repair processes [379]. Thus when neratinib was pre-administered, it produced HER receptor blockade, which might enhance cisplatin-stimulated cytotoxicity [342].

When the TKIs were combined with carboplatin, they did not show any clear favourable results. This was also seen before in a platinum-sensitive recurrent ovarian cancer clinical trial, whereby lapatinib plus carboplatin showed no encouraging effects, and patients suffered from increased toxicity [288]. However, another ovarian cancer study evaluating the combination of carboplatin/paclitaxel with lapatinib in

stage III or IV relapsed patients, had positive results and toxicity was not a limitation [358, 380]. The estimated OS of relapsed ovarian cancer patients receiving the combination treatment was 16 months in the platinum-sensitive patients, as opposed to 39 months in the platinum-resistant patients [380].

The co-administration of paclitaxel and lapatinib in PEA1 cells gave very promising synergistic results, as opposed to the additive effects obtained when lapatinib was administered in PEA2 cells. This might suggest that paclitaxel sensitizes cells to lapatinib treatment, as was also noticed in a clinical trial whereby lapatinib was combined with paclitaxel and carboplatin [380]. Previous studies in HER2-positive gastric cancer revealed that this combination gave better efficacy in IHC3+ patients, with median OS of 11 months [381]. Combining neratinib with paclitaxel resulted predominantly in additive effects in the PEA1 and PEA2 ovarian cancer cell lines. Pre-clinical and clinical trial breast cancer studies show that co-administration of neratinib and paclitaxel have a favourable clinical efficacy, despite the side effects [304, 382, 383].

Lapatinib and neratinib were able to sensitize the ovarian cancer cell lines to pertuzumab. PEA1 cells were not very sensitive to lapatinib treatment, however the addition of pertuzumab increased the anti-proliferative effect of lapatinib, supported by results gathered from lapatinib-resistant breast cancer cell lines [384]. Neratinib has previously been investigated in combination with trastuzumab in breast cancer, and it produced better inhibitory effects than either agent alone, even in trastuzumab-resistant tumours [299]. Similarly, lapatinib combined with trastuzumab had enhanced inhibitory effects in breast cancer [385] and trastuzumab/pertuzumab-resistant colorectal cancer [386]. In this experiment, neratinib was able to produce an additive inhibitory effect with trastuzumab in the PEA1 cell line, a cell line which is not very responsive to trastuzumab. However, unlike previous studies, lapatinib did not give additive or synergistic effects when combined with trastuzumab in the ovarian cancer cell lines investigated, which might be because the cell lines investigated are not HER2-amplified.

Although these combinations show promise, further experiments with different concentration, schedules and *in vivo* testing are required. It is imperative to make sure that the combination treatment doses are safe *in vivo* and that the benefits from prolongation of life out-way the risks.

3.7.3 TKIs reduce adhesion, migration and invasion in all ovarian cancer cell lines

Metastasis forms one of the hallmarks of cancer [5]. This process involves the shedding of cells from the primary tumour, invasion to surrounding tissue, migration to other sites, and finally adhesion to new sites, which is often the peritoneal cavity in the case of ovarian cancer. This process is very frequent in HGSOC [387]. Metastasis can be driven by the HER pathway, thus inhibiting this signalling by use of TKIs or mAbs should diminish this process. Therefore, to test this hypothesis, the TKIs and mAbs were used in experiments to assess their activity against migration, adhesion and invasion in the ovarian cancer cell lines.

The cell lines were first grown as spheroids to mimic the *in vitro* hypoxic and cell-cell interaction environment. The only cell line which did not grow spheroids was the PEA1 cell line. Generally, the higher the EGFR, HER2 and HER3 expression, the better the invasion ability of the cells, which was also found in other studies [388-391]. When neratinib was added to the spheroids, these reduced their invading abilities, supporting neratinib as an anti-invasive agent.

Overexpression of EGFR and HER2 have been found to promote breast cancer cellular migration [392, 393]. However, in this study the higher the EGFR expression level, the lower the rate of migration. With respect to HER2, there was no obvious relationship between HER2 expression and migration.

Previous studies have shown how TKIs inhibit migration of cancer cells [272, 394], however this notion has not been studied with all the TKIs investigated in this study. Therefore, a scratch assay was performed on all cell lines when they were 100% confluent and the percentage migrated by each cell line was investigated. All TKIs

were able to inhibit migration, however, sapitinib performed better than the others overall, followed by canertinib; both are reversible TKIs (Figure 3.19). The platinum-resistant cell lines, PEA2 and PEO23, were more sensitive to the anti-migratory effects of the TKIs. However, the other platinum-resistant cell line PEO4 was not as sensitive to the TKIs, similar to the results obtained from proliferation experiments. Overall, PEO4 was the least sensitive cell line, which interestingly ties with its lower migratory rates. Additionally, the cell lines which had higher migratory rates from each pair, were more sensitive to the TKI anti-migratory effects than their counterpart cell line. This suggests that TKIs are more effective against more rapidly migrating cells.

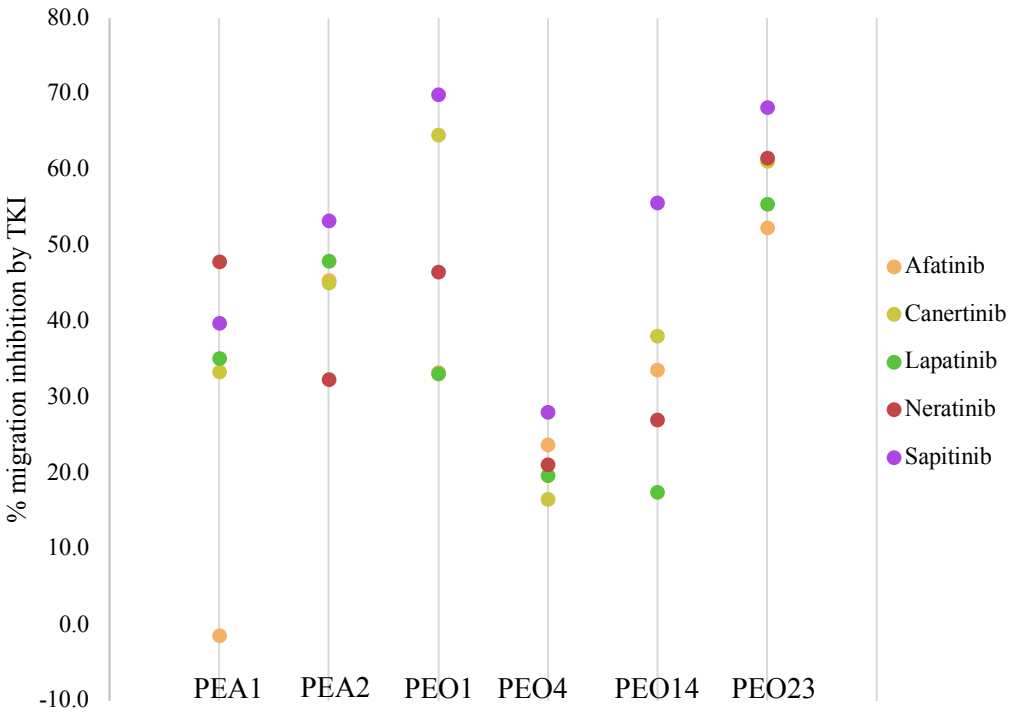


Figure 3.19: Illustration of the percentage migration inhibitory effect of the 5 TKIs on each cell line

The mAbs were also assessed for their inhibitory effects on the ovarian cancer cell lines. Only the PEA1-PEA2 pair was studied as it was the most migratory. Here, the mAbs had an anti-migratory effect on the PEA2 cell line, and a minimal effect on the PEA1 cell line. This could be attributed to the fact that PEA2 cell line had more HER2 expression than the PEA1 cell line. The combination of mAbs, (which consisted of 25 µg/mL trastuzumab + 25 µg/mL pertuzumab) produced the best anti-migratory effects,

especially in PEA2 cell line. This contrasts with the mAb effect on proliferation, where pertuzumab showed more beneficial effects.

Following adhesion, tumour cells often release proteolytic enzymes which start degrading ECM molecules to give way to invasion into proteolytic tissue [395]. *In vitro* studies indicate that collagen has better adhesive effects for ovarian cancer cell lines than other ECM proteins [396, 397]. This was also noticed in this study, whereby cells adhered better to collagen than fibronectin or laminin. The effect of neratinib on adhesion was assessed by pre-incubating cells with neratinib and adding neratinib in the wells. Neratinib was able to reduce adhesion of almost all the cell lines, except for collagen and laminin in PEO14. Intriguingly, PEO14 was also resistant to neratinib anti-proliferative treatment. Studies suggest a link between integrins and activation of the MAPK pathway [398], which could suggest that the PEO14 cell line's lack of sensitivity towards neratinib could be because it is unable to inhibit the MAPK pathway.

In conclusion, the TKIs inhibit proliferation, migration, adhesion and invasion of these cell lines, irrespective of their platinum sensitivity. Although mAbs have a role in the reduction of proliferation and migration, they are not as effective in these ovarian cancer cell lines as the TKIs under investigation, even though their clinical target concentration has been met. Furthermore, the functional assays revealed a trend that suggested that the higher the HER receptor expression, the better the response to the HER-targeted therapies. Selected combinations of inhibitors were able to increase potency and these combinations included neratinib with cisplatin, lapatinib with paclitaxel and pertuzumab with neratinib/lapatinib.

4 The response of HER-targeted therapy to EGF and HRG modulation in ovarian cancer cell lines

EGF and HRG are ligands found in varying concentrations within ovarian cancers and act on the HER pathway receptors, causing receptor dimerisation [139]. The dimerised receptors elicit signalling transduction, which leads to cellular proliferation and resistance to apoptosis [147]. These two ligands were chosen for further investigation as they differentially stimulate the HER family receptors, with EGF activating EGFR, whilst HRG activates the HER3 and HER4 receptors. From the previous chapter, the HER family expression was shown to play an important role in the efficacy of the TKIs and mAbs, and this chapter seeks to determine whether ligand activation is as important in the efficacy of inhibitors.

This chapter describes studies that explored how the platinum-sensitive and platinum-resistant HGSOC cell lines respond to EGF and HRG. The effects of the growth factors on cell signalling and functionality were investigated in the absence and presence of TKIs and mAbs.

Additionally, the effect of TKIs and mAbs on the suppression of the response that these ligands bring about in the ovarian cancer cell lines was investigated. This may help define which tumour characteristics influence response to these agents.

4.1 Time course effects of EGF and HRG modulation on HER receptors

The effects of EGF and HRG on cell signalling in the cell lines over a period of 48 h were first investigated. Cells were exposed to 1 ng/mL EGF or 1 ng/mL HRG for 24 h or 48 h before collecting lysates. These concentrations of EGF and HRG are typical concentrations found in cancer patients [399-403] and have been widely used in previous *in vitro* studies [404, 405]. Neratinib (0.1 μM) was also added for the same time period to assess its effect on ligand activation. Neratinib was the TKI chosen since the cell lines responded well in terms of proliferation and within therapeutic concentrations. Cells were maintained in 0.1% FBS, since FBS might contain these growth factors in unknown amounts. The cells were not completely serum starved so they would not struggle to grow. Figure 4.1 represents western blots obtained for PEA1-PEA2 cell lines, Figure 4.2 for PEO1-PEO4 cell lines, and Figure 4.3 for PEO14-PEO23 cell lines. The effects of EGF and HRG on the downstream signalling pathways PI3K/Akt/mTOR and Ras-Raf-MEK-ERK were indicated by increased expression of p-Akt (Ser473) and p-ERK (Thr202/Tyr204) respectively.

Addition of EGF increased the expression of HER2 and HER3 in PEA1 and PEA2 cells and HER4 in only PEA2 cells after 24 h and 48 h, whilst expression of EGFR decreased (Figure 4.1). Similarly, addition of HRG increased expression of HER2, at 48 h in both cell lines, while the expression of EGFR, HER3 and HER4 remained relatively unchanged. The addition of neratinib reduced HER2 expression at both time points in both cell lines, and HER4 expression at all time points and with EGF and HRG in PEA2. p-Akt was stimulated in PEA2 upon addition of EGF after 48 h and upon addition of HRG at 24 h and 48 h. Similarly, p-ERK was stimulated in both cell lines, at both time points with EGF as well as HRG. Neratinib reduced the Akt and ERK phosphorylation in all circumstances, except p-Akt when HRG was added to PEA2 cells.

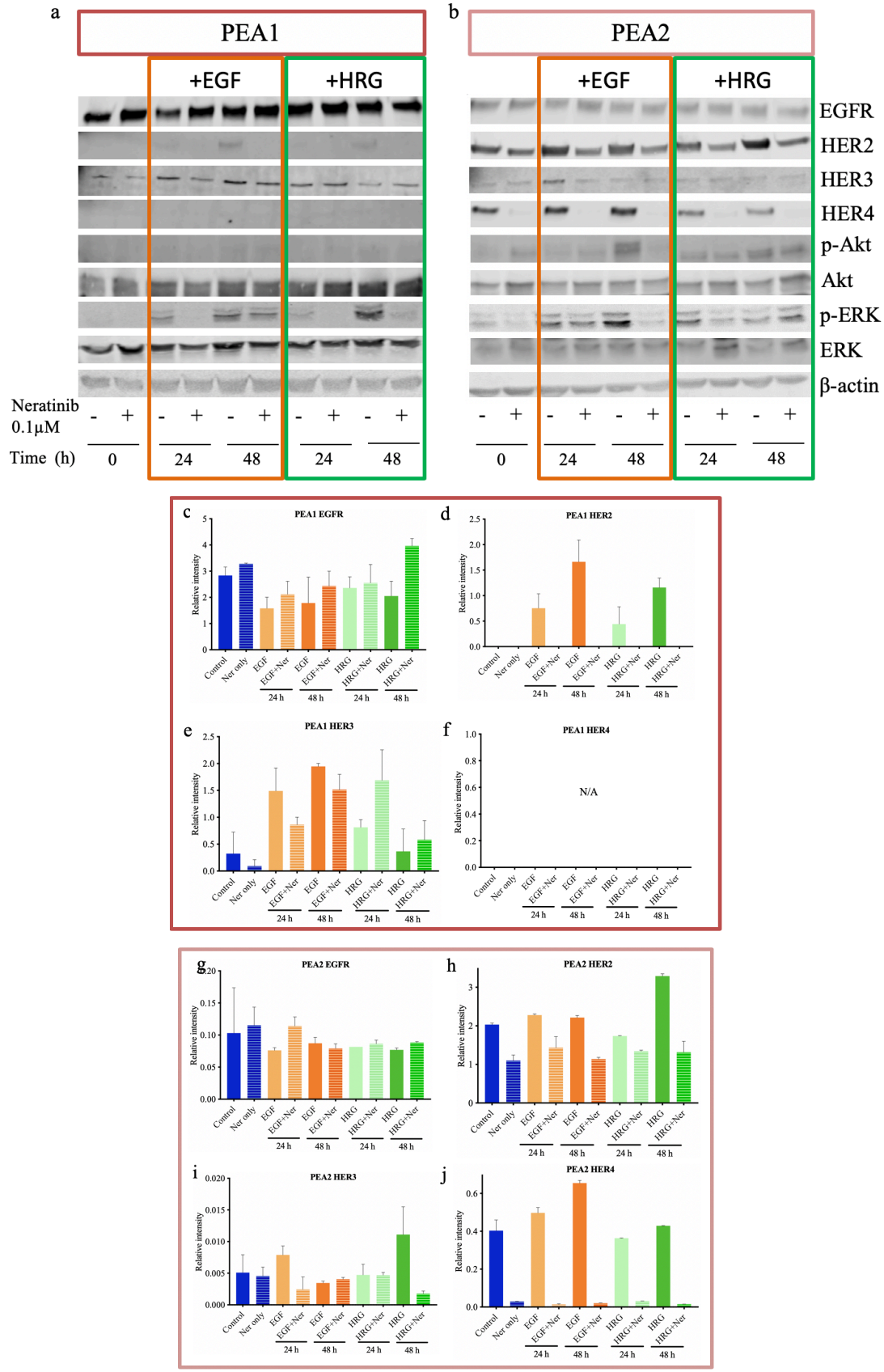


Figure 4.1: HER protein expression and downstream signalling proteins for PEA1-PEA2 when EGF/HRG with or without neratinib were added

(a and b) western blots of the HER receptors and downstream signalling effects of EGF/HRG ± neratinib for (a) PEA1 cell line, or (b) PEA2 cell line, (c-j) Bar graphs of HER expression normalised to loading control in PEA1 for (c) EGFR, (d) HER2, (e) HER3 and (f) HER4 and in PEA2 for (g) EGFR, (h) HER2, (i) HER3 and (j) HER4. EGF or HRG with or without 0.1μM neratinib were added to the cells for 24 h or 48 h. The experiment was repeated twice and the results are presented as mean of the relative intensity and error bars show standard deviation. The western blots are a representative of the repeated results, and the loading controls are representative of the other membranes. N/A means not applicable.

In PEO1 and PEO4 cells, addition of EGF or HRG had little effect on EGFR, HER2, HER3 expression (Figure 4.2). However, 48 h following EGF addition, HER4 expression increased in both cell lines. When neratinib was added to the cells, it decreased HER4 expression in EGF-stimulated PEO1 cells and HER2 and HER4 expression in EGF and HRG-induced PEO4 cells. The addition of EGF and HRG for 24 h and 48 h did not affect Akt phosphorylation, whereas addition of EGF increased ERK phosphorylation after 24 h and further stimulated phosphorylation after 48 h in both cell lines. HRG only stimulated p-ERK in PEO4 cells after 48 h. Neratinib inhibited the phosphorylation of ERK, except when HRG was added for 48 h in the PEO1 cell line.

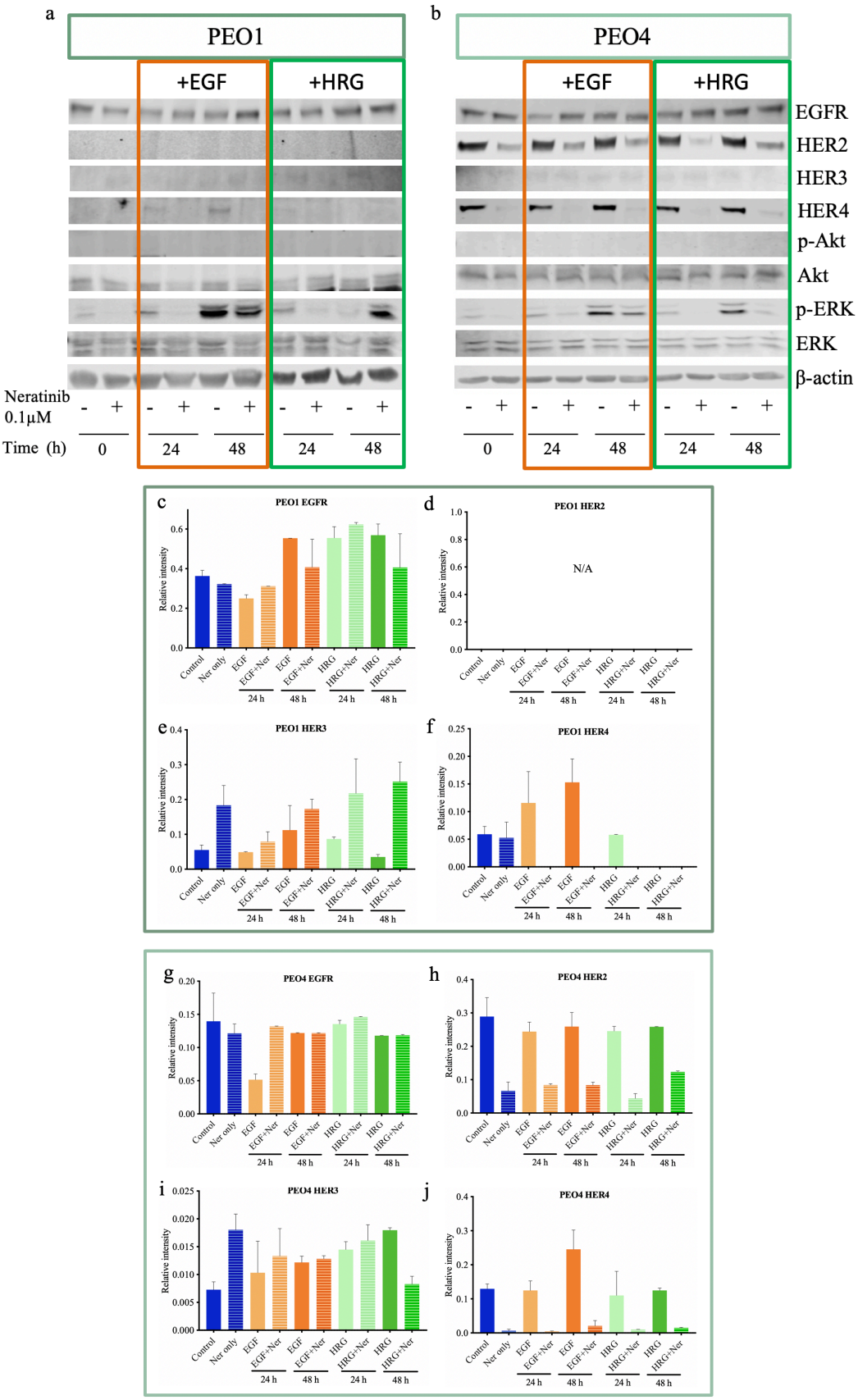


Figure 4.2: HER protein expression and downstream signalling proteins for PEO1-PEO4 when EGF/HRG with or without neratinib were added

(a and b) western blots of the HER receptors and downstream signalling effects of EGF/HRG ± neratinib for (a) PEO1 cell line, or (b) PEO4 cell line, (c-j) Bar graphs of HER expression normalised to loading control in PEO1 for (c) EGFR, (d) HER2, (e) HER3 and (f) HER4 and in PEO4 for (g) EGFR, (h) HER2, (i) HER3 and (j) HER4. EGF or HRG with or without 0.1μM neratinib were added to the cells for 24 h or 48 h. The experiment was repeated twice and the results are presented as mean of the relative intensity and error bars show standard deviation. The western blots are a representative of the repeated results, and the loading controls are representative of the other membranes. N/A means not applicable.

For the PEO14 cell line, EGFR, HER2, and HER4 expression remained unchanged after EGF and HRG addition, while HER3 expression decreased for the PEO23 cell line (Figure 4.3). HER2 expression increased 48 h after addition of EGF and after 24 h and 48 h addition of HRG. Neratinib reversed these HER2 stimulations. P-Akt in both cell lines remained unaffected upon the different treatments. The addition of EGF and HRG did not increase the baseline phosphorylation of ERK in PEO14 cells, however neratinib inhibited baseline phosphorylation. In PEO23 cells EGF and HRG increased p-ERK expression after 48 h, which was not inhibited by neratinib.

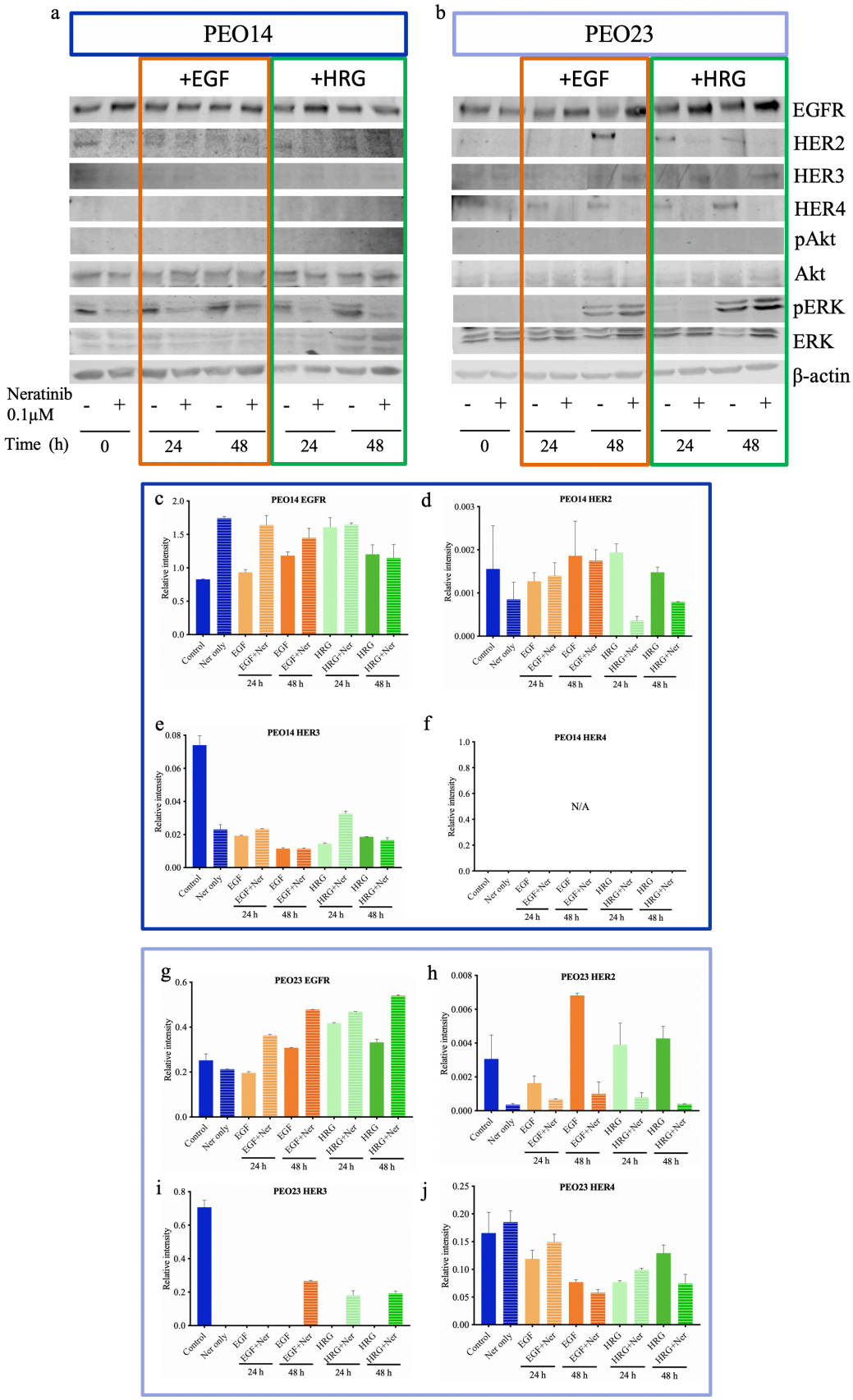


Figure 4.3: HER protein expression and downstream signalling proteins for PEO14-PEO23 when EGF/HRG with or without neratinib were added

(a and b) western blots of the HER receptors and downstream signalling effects of EGF/HRG ± neratinib for (a) PEO14 cell line, or (b) PEO23 cell line, (c-j) Bar graphs of HER expression normalised to loading control in PEO14 for (c) EGFR, (d) HER2, (e) HER3 and (f) HER4 and in PEO23 for (g) EGFR, (h) HER2, (i) HER3 and (j) HER4. EGF or HRG with or without 0.1μM neratinib were added to the cells for 24 h or 48 h. The experiment was repeated twice and the results are presented as mean of the relative intensity and error bars show standard deviation. The western blots are a representative of the repeated results, and the loading controls are representative of the other membranes. N/A means not applicable.

4.2 Downstream pathway response to HER-targeted therapy with EGF and HRG modulation

The cell lines were treated with TKIs or mAbs, and subsequently EGF or HRG were added to investigate whether the HER-targeted therapy would be able to suppress pathway stimulation in the presence of EGF/HRG modulation. p-EGFR (Tyr1289) and p-HER2 (Tyr1221/1222) expression were evaluated as indicators of EGFR and HER2 activation respectively. Similar to experiments described in section 4.1, phosphorylation of the downstream products Akt and ERK were monitored to evaluate the response of these pathways to HER-targeted therapy.

4.2.1 TKIs with EGF and HRG modulation

The cell lines were first treated with pharmacologically achievable concentrations of the TKIs (afatinib 0.08 μM, canertinib 1 μM, lapatinib 1.5 μM, neratinib 0.1 μM, sapitinib 1 μM) [307, 310, 312, 314, 361] for 24 h in media containing 0.1% FBS. Subsequently, 1 ng/mL EGF or 1 ng/mL HRG were added 30 min before lysate collection. Figure 4.4 represents the western blots obtained for PEA1-PEA2 cell lines, Figure 4.5 for PEO1-PEO4 cell lines, while Figure 4.6 represents PEO14-PEO23 cell lines.

EGF and HRG stimulation in the PEA2 cell line increased EGFR phosphorylation (Figure 4.4). All TKIs tested reduced this growth factor stimulated phosphorylation. Total EGFR expression remained unchanged at this time point. Phosphorylated HER2 did not increase with the addition of EGF or HRG but all TKIs inhibited p-HER2 in the PEA2 cell line, and all except for lapatinib in EGF and HRG stimulated PEA1 cells. Total HER2 expression increased in PEA1 cells after the addition of EGF and

HRG, and was reduced by the TKIs. Afatinib, canertinib, and neratinib caused a modest decrease in total HER2 in PEA2 cells.

In PEA1 cells, HRG stimulated a small increase in p-Akt, which was reduced by the TKIs. In contrast, there were no changes to p-Akt expression in PEA2 cells. Phosphorylation of ERK was stimulated by EGF and HRG in both cell lines. The TKIs reversed the phosphorylation in EGF-stimulated PEA1. In HRG-stimulated PEA1 cells, this p-ERK increase was partially reversed. In EGF-stimulated PEA2 cells, the phosphorylation was unaffected by TKIs, while the HRG-stimulated cells had their phosphorylation decreased by the TKIs.

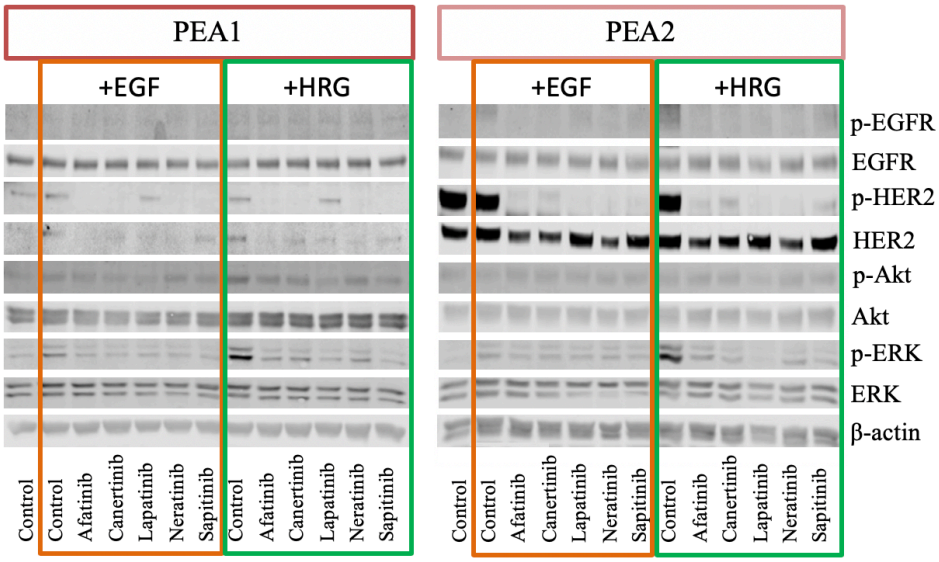


Figure 4.4: HER pathway signalling proteins for PEA1 and PEA2 cell lines when EGF/HRG with or without TKIs were added

TKIs were added at clinically achievable concentrations for 24 h, and 30 min before lysate collection, 1 ng/mL EGF or HRG were added to the cells. The experiment was repeated twice and the results show a representative of the results obtained for the western blots, and the loading controls are representative of the other membranes.

Addition of EGF and HRG to the PEO4 cell line, caused phosphorylation of EGFR (Figure 4.5). Afatinib, canertinib and neratinib (but not lapatinib or sapitinib) inhibited EGF-driven EGFR phosphorylation, while all TKIs apart from sapitinib reduced HRG-stimulated EGFR phosphorylation. Although PEO1 cells expressed low levels of total HER2, p-HER2 expression was present in the unstimulated cells. This basal level of phosphorylation was inhibited by all TKIs. In PEO4 cells, addition of EGF and HRG caused phosphorylation of HER2, which was inhibited by the TKIs. Total HER2

expression was reduced in afatinib and neratinib treated EGF and HRG stimulated PEO4 cells.

In PEO1 cells, phosphorylated Akt increased when EGF and HRG were added. All the TKIs, except canertinib in EGF-stimulated cells, were able to reduce the phosphorylation. Conversely, there was no detectable active p-Akt in PEO4 cells. In PEO1 cells, p-ERK increased with EGF and HRG stimulation, which was decreased in the presence of the TKIs. PEO4 demonstrated an increase in p-ERK with the addition of HRG, which was only partially inhibited by the TKIs.

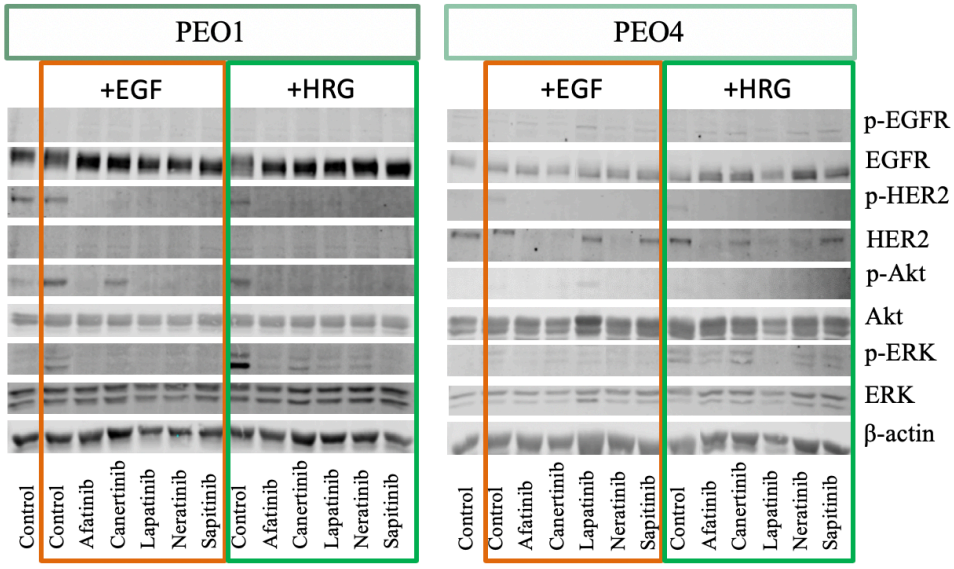


Figure 4.5: HER pathway signalling proteins for PEO1 and PEO4 cell lines when EGF/HRG with or without TKIs were added

TKIs were added at clinically achievable concentrations for 24 h, and 30 min before lysate collection, 1 ng/mL EGF or HRG were added to the cells. The experiment was repeated twice and the results show a representative of the results obtained for the western blots, and the loading controls are representative of the other membranes.

PEO14 cells have basal EGFR phosphorylation, which was not affected by the addition of EGF and HRG (Figure 4.6). This phosphorylation, was reduced by afatinib, neratinib and sapitinib. In the PEO23 cell line, EGF and HRG stimulated p-EGFR. All TKIs reduced this phosphorylation, especially afatinib, canertinib and neratinib. All TKIs except lapatinib reduced p-HER2 in EGF-stimulated PEO14 cells, while all TKIs reduced p-HER2 in HRG-stimulated PEO14 cells. In PEO23 cells, all TKIs apart from canertinib reduced p-HER2. Total HER2 remained the same in PEO14 cells, however it decreased in all TKI-treated PEO23 cell lines.

In PEO23 cells, p-Akt was stimulated on addition of EGF and HRG. All TKIs were able to reduce the phosphorylation, apart from canertinib in EGF-stimulated PEO23 cells. Phosphorylated ERK was stimulated on addition of HRG to PEO14 cells. Afatinib and sapitinib were the TKIs best able to reduce this phosphorylation. In PEO23 cells, ERK was phosphorylated upon the addition of EGF and HRG, which was inhibited in the presence of all the TKIs.

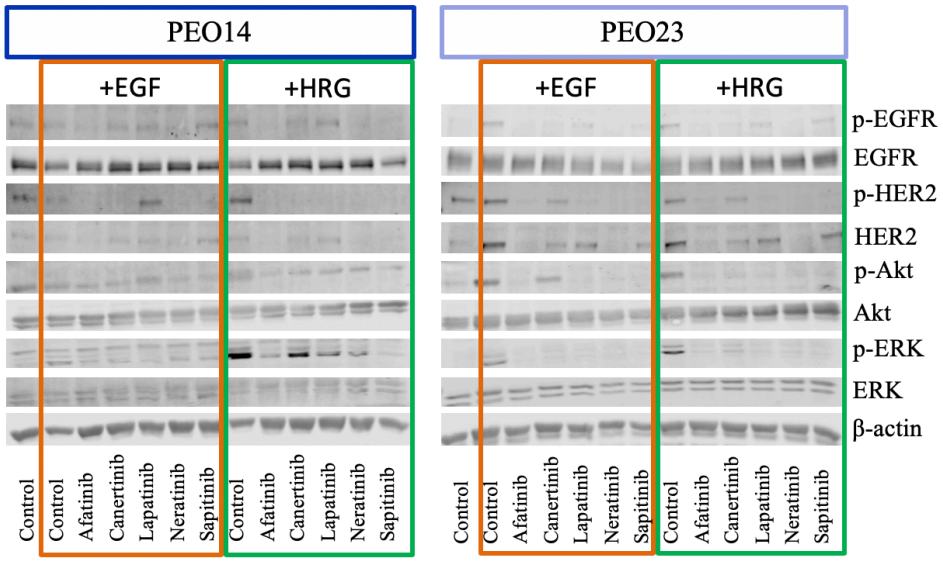


Figure 4.6: HER pathway signalling proteins for PEO14 and PEO23 cell lines when EGF/HRG with or without TKIs were added

TKIs were added at clinically achievable concentrations for 24 h, and 30 min before lysate collection, 1 ng/mL EGF or HRG were added to the cells. The experiment was repeated twice and the results show a representative of the results obtained for the western blots, and the loading controls are representative of the other membranes.

4.2.2 The effects of mAbs on EGF and HRG modulation

The six cell lines were treated with 50 μg/mL pertuzumab, 50 μg/mL trastuzumab or 25 μg/mL + 25 μg/mL of the combination for 24 h in 0.1% FBS media. Following this incubation, 1 ng/mL EGF or 1 ng/mL HRG were added 30 min before lysate collection. The western blots for all the cell lines are illustrated in Figure 4.7.

With prior addition of the mAbs, p-Akt expression in the EGF-stimulated cell lines was not reduced, while in all cell lines apart from PEO4, p-Akt increased. Similarly, p-ERK was increased in all cell lines, except PEO4 upon the addition of EGF. The mAbs were not able to reverse the p-ERK when this was induced by EGF.

Both PE04 and PE023 cells had enhancement of p-Akt when HRG was added to the cells. All mAbs were able to reduce this phosphorylation in PE023 cells and pertuzumab and the combination in PE04 cells. HRG produced a marked increase in ERK phosphorylation in all cell lines, which was reversed by the mAbs under investigation. Total Akt and ERK expression levels remained the same throughout.

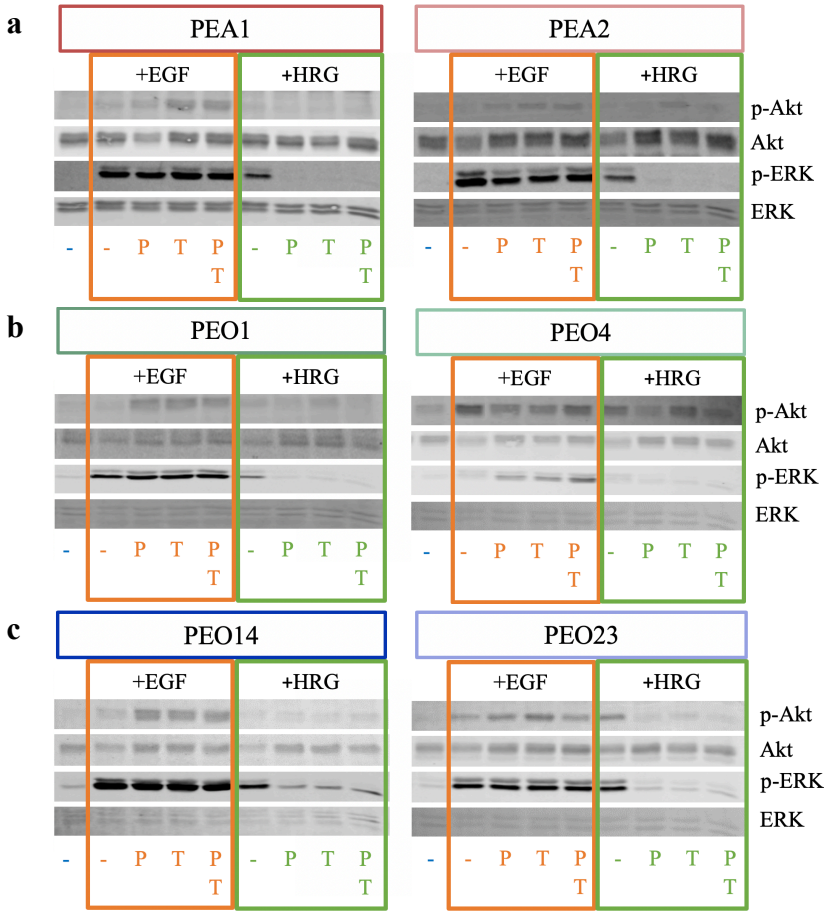


Figure 4.7: HER pathway signalling proteins for the cell lines when EGF/HRG with or without mAbs were added

Western blots for (a) PEA1-PEA2 cell lines, (b) PEO1-PEO4 cell lines, and (c) PEO14-PEO23 cell lines. mAbs were added at clinically achievable concentrations for 24 h, and 30 min before lysate collection, 1 ng/mL EGF or HRG were added to the cells. P means pertuzumab; T means trastuzumab. The experiment was repeated twice and the results show a representative of the results obtained for the western blots.

4.2.3 Time course effect of neratinib with EGF and HRG

The downstream signalling changes after addition of EGF and HRG over a period of 60 min were analysed by western blot. This was investigated by adding 0.1 μM neratinib simultaneously with 1 ng/mL EGF or 1 ng/mL HRG or 1 ng/mL of both. The

time points chosen were 5 min, 10 min, 15 min, 30 min and 60 min. The cells were serum deprived (0.1% FBS in medium) for 2 h prior to the addition of growth factors at each time point.

For PEA1 cells, the addition of EGF or HRG individually had no effect on p-Akt expression (Figure 4.8). Combination of EGF and HRG caused a stimulation in p-Akt as early as 5 min with a subsequent reduction of expression. Neratinib inhibited this stimulation to some degree. P-ERK stimulation occurred with EGF addition at 5 min, and was sustained up to 30 min. Neratinib inhibited the amplitude of this phosphorylation as early as 5 min. The addition of HRG caused a modest increase in phosphorylation of ERK, which peaked at 30 min. Addition of neratinib inhibited this phosphorylation, especially at 30 min. When EGF and HRG were added simultaneously, the phosphorylation increased gradually from 5 min to 15 min and started decreasing by 30 min. When neratinib was added to the combination, it was ineffective at decreasing ERK phosphorylation.

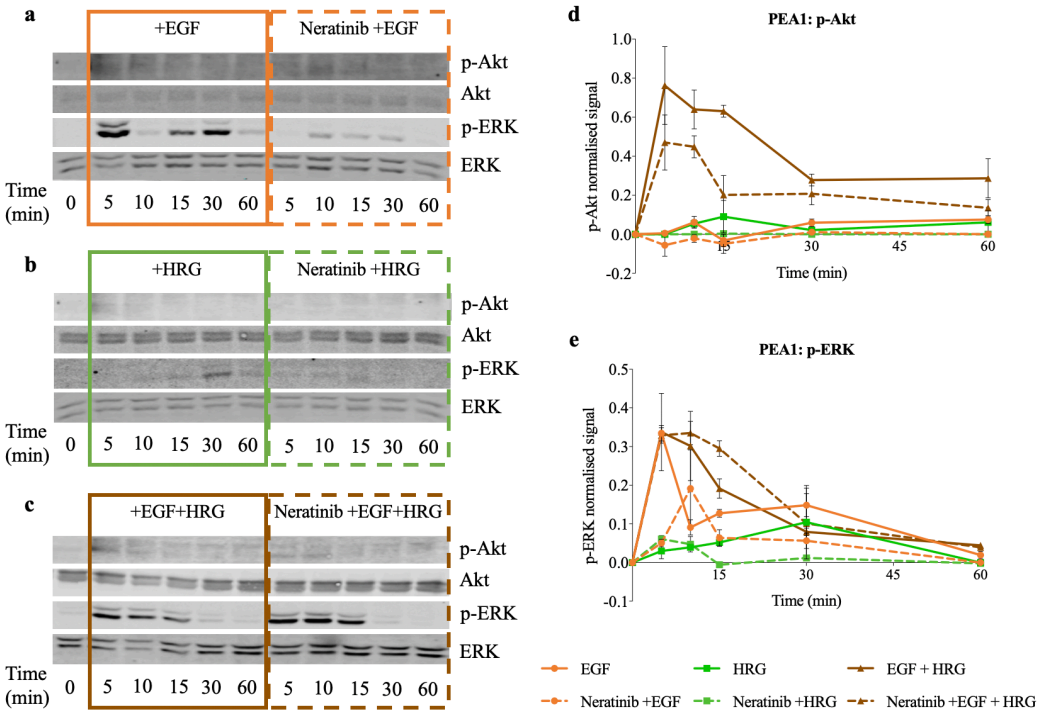


Figure 4.8: HER pathway signalling proteins for PEA1 cell line when EGF/HRG with or without neratinib was added

Western blots for (a) PEA1 with 1 ng/mL EGF stimulation ± neratinib, (b) PEA1 with 1 ng/mL HRG stimulation ± neratinib (c) PEA1 with EGF and HRG stimulation ± neratinib. Graphs representing the normalised (d) p-Akt expression with Akt from the induction of the control p-

Akt (0 min), and (e) p-ERK expression with ERK from the induction of the control p-ERK (0 min). The experiment was repeated twice and the results show a representative of the results obtained for the western blots. Error bars represent standard deviation.

In the PEA2 cell line (Figure 4.9), the addition of EGF and HRG separately caused initial modest phosphorylation of Akt at 5 min which, decreased by 15 min. When EGF and HRG were added simultaneously, p-Akt peaked at 10 min, and gradually started to decrease. Neratinib had no effect on this. Phosphorylated ERK expression was increased by both ligands, alone and simultaneously. Expression of p-ERK peaked at 5 min and afterwards the phosphorylation gradually decreased, until 60 min. As for p-Akt, neratinib had no effect on these expression changes in this cell line.

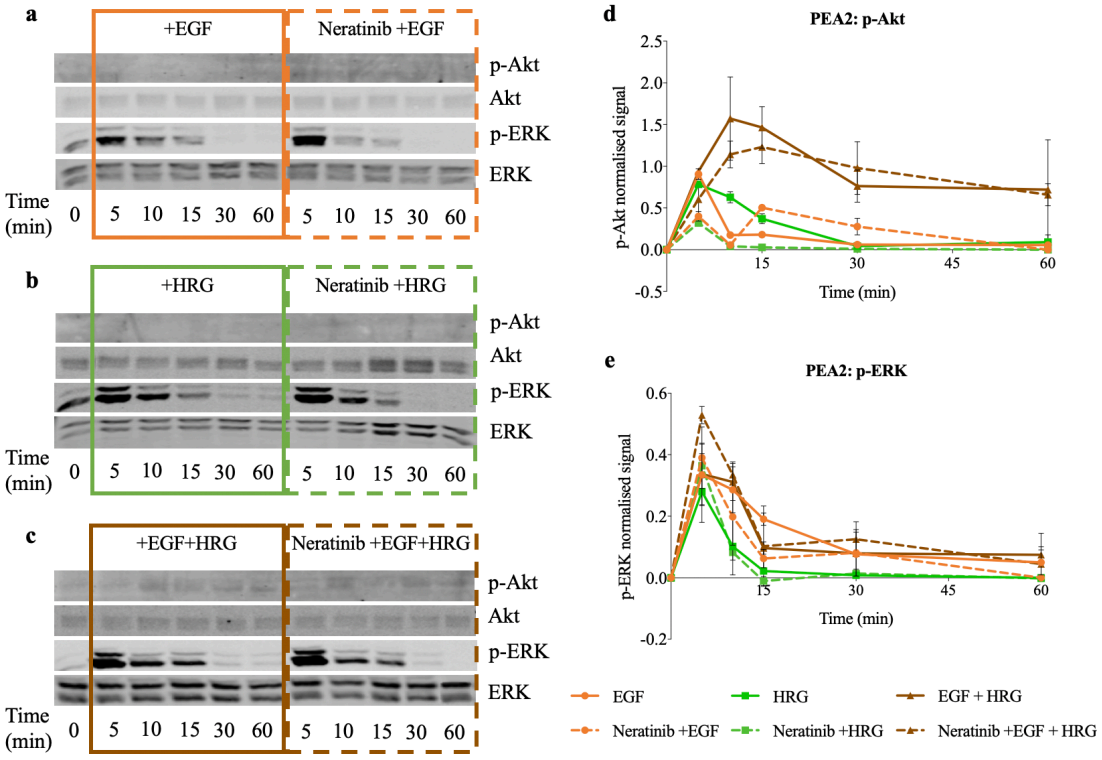


Figure 4.9: HER pathway signalling proteins for PEA2 cell line when EGF/HRG with or without neratinib was added

Western blots for (a) PEA2 with 1 ng/mL EGF stimulation ± neratinib, (b) PEA2 with 1 ng/mL HRG stimulation ± neratinib (c) PEA2 with EGF and HRG stimulation ± neratinib. Graphs representing the normalised (d) p-Akt expression with Akt from the induction of the control p-Akt (0 min), and (e) p-ERK expression with ERK from the induction of the control p-ERK (0 min). The experiment was repeated twice and the results show a representative of the results obtained for the western blots. Error bars represent standard deviation.

Upon the addition of EGF, HRG and their combination to the PEO1 cell line, p-Akt was stimulated after 5 min, (Figure 4.10). The highest stimulations generally occurred

when EGF and HRG were administered concurrently When neratinib was administered simultaneously, it reversed the effects of the HRG and the combination of ligands. The ligands caused phosphorylation of ERK, as early as 5 min, which peaked at 10-15 min and gradually decreased until the 60th min. The combination peaked at 10 min, after which it steadily decreased. EGF and the combination of EGF and HRG caused the greatest stimulation of p-ERK. When neratinib was added to either EGF or HRG alone, it decreased the amplitude of the phosphorylation at all time points. However, when neratinib was added to the combination of ligands, p-ERK expression was not reduced.

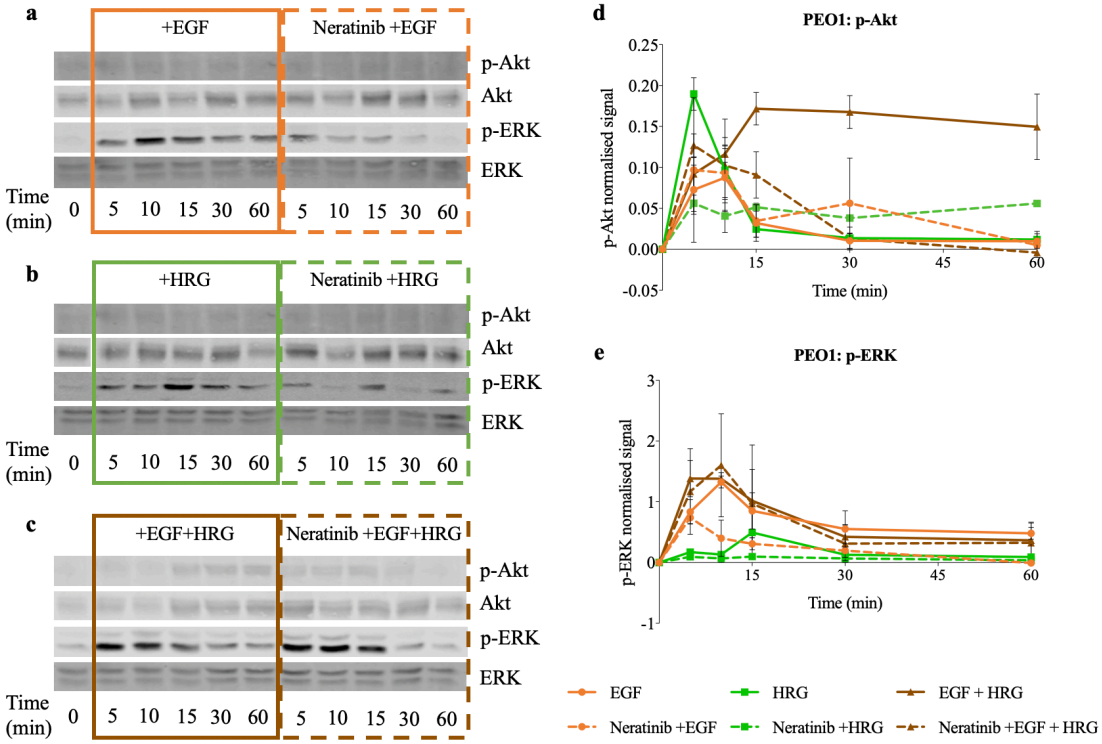


Figure 4.10: HER pathway signalling proteins for PEO1 cell line when EGF/HRG with or without neratinib was added

Western blots for (a) PEO1 with 1 ng/mL EGF stimulation ± neratinib, (b) PEO1 with 1 ng/mL HRG stimulation ± neratinib (c) PEO1 with EGF and HRG stimulation ± neratinib. Graphs representing the normalised (d) p-Akt expression with Akt from the induction of the control p-Akt (0 min), and (e) p-ERK expression with ERK from the induction of the control p-ERK (0 min). The experiment was repeated twice and the results show a representative of the results obtained for the western blots. Error bars represent standard deviation.

In PEO4 cells, ligands increased p-Akt to different extents (Figure 4.11). Increased phosphorylation occurred by 5 min, and in EGF-stimulated cells, this peaked at 15 min. Neratinib had little effect on this. When EGF, HRG and the combination were

added to the PEO4 cell line, they caused increased phosphorylation of p-ERK, even at 5 min. With EGF, the phosphorylation peaked at 10 min. In all cases, the phosphorylation decreased rapidly by 15 min. The addition of neratinib did not diminish this activation.

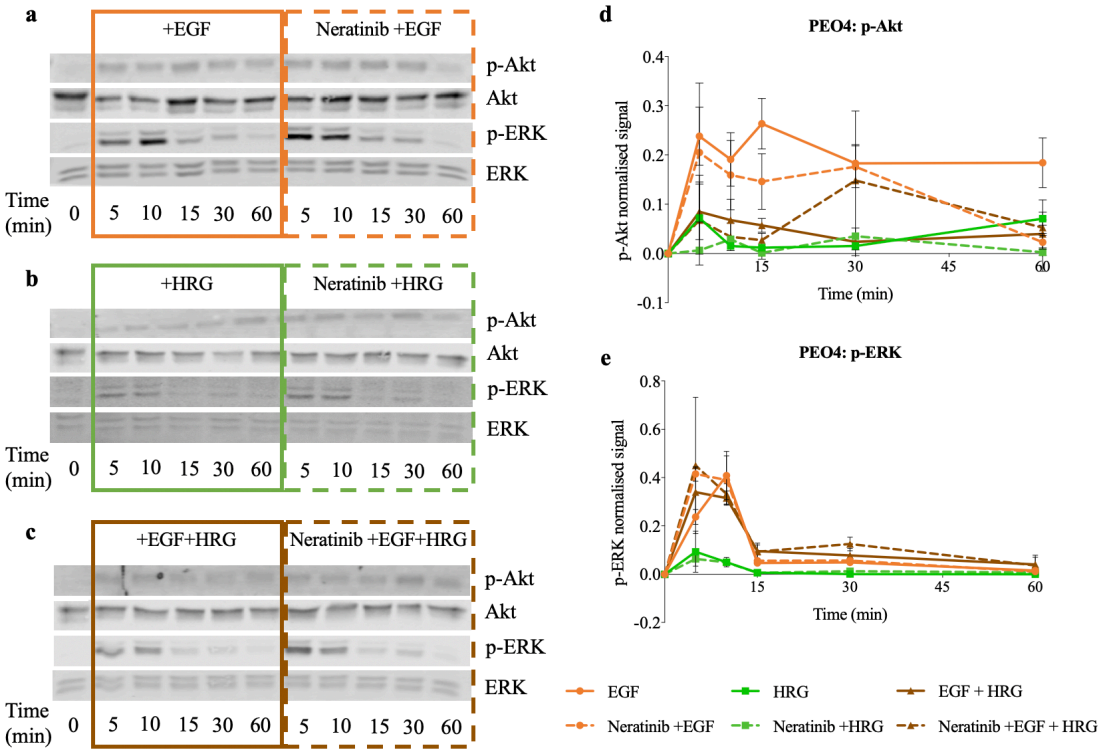


Figure 4.11: HER pathway signalling proteins for PEO4 cell line when EGF/HRG with or without neratinib was added

Western blots for (a) PEO4 with 1 ng/mL EGF stimulation ± neratinib, (b) PEO4 with 1 ng/mL HRG stimulation ± neratinib (c) PEO4 with EGF and HRG stimulation ± neratinib. Graphs representing the normalised (d) p-Akt expression with Akt from the induction of the control p-Akt (0 min), and (e) p-ERK expression with ERK from the induction of the control p-ERK (0 min). The experiment was repeated twice and the results show a representative of the results obtained for the western blots. Error bars represent standard deviation.

In the PEO14 cell line (Figure 4.12), addition of EGF and the combination of EGF and HRG increased phosphorylation of Akt to similar extents, while the presence of HRG alone produced limited stimulation. The addition of neratinib to all three scenarios fully inhibited the ligand-activated phosphorylation of Akt, as early as from the first 5 min. ERK phosphorylation was increased by all three growth factor additions. Increase in phosphorylation was observed at 5 min, and continued to increase up to 15 min. After that, the p-ERK remained steady for the remaining 45 min. When neratinib was added, it inhibited the phosphorylation of ERK in all the three settings.

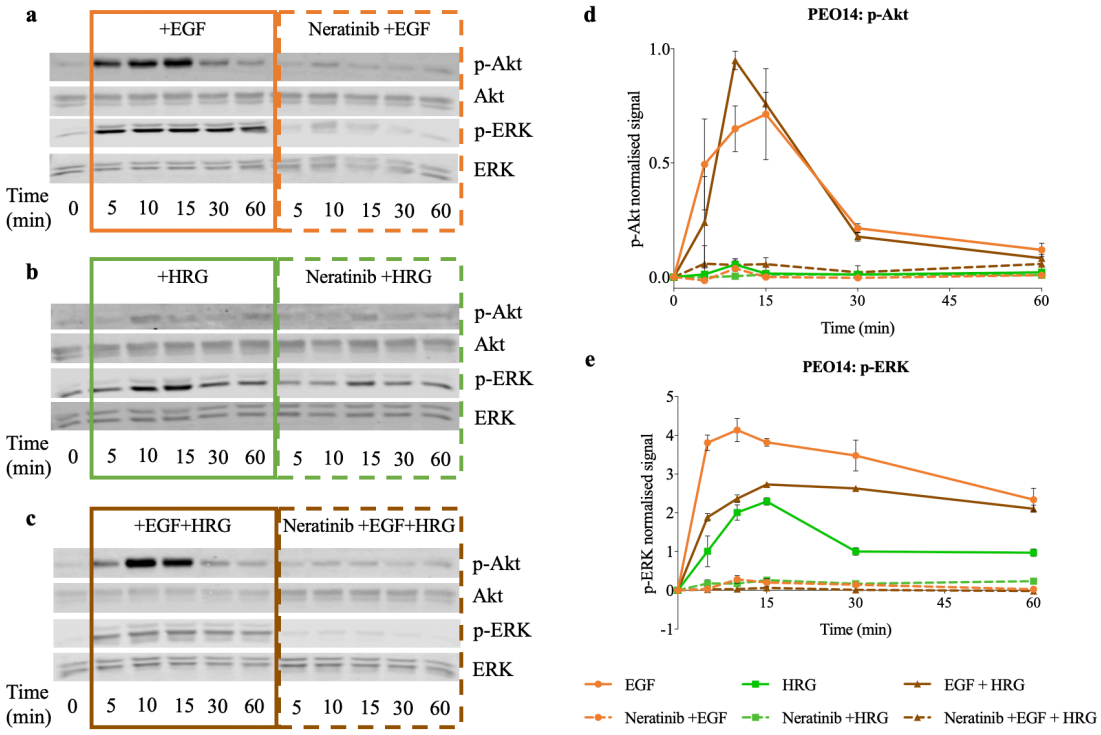


Figure 4.12: HER pathway signalling proteins for PEO14 cell line when EGF/HRG with or without neratinib was added

Western blots for (a) PEO14 with 1 ng/mL EGF stimulation ± neratinib, (b) PEO14 with 1 ng/mL HRG stimulation ± neratinib (c) PEO14 with EGF and HRG stimulation ± neratinib. Graphs representing the normalised (d) p-Akt expression with Akt from the induction of the control p-Akt (0 min), and (e) p-ERK expression with ERK from the induction of the control p-ERK (0 min). The experiment was repeated twice and the results show a representative of the results obtained for the western blots. Error bars represent standard deviation.

Akt was phosphorylated in PEO23 cells when EGF, HRG and their combination was added to the cells, which peaked at 15 min (Figure 4.13). Expression gradually reduced during the following 45 min. Upon the addition of neratinib, p-Akt phosphorylation was completely inhibited at all time points. Addition of EGF with HRG to PEO23 cells caused the greatest ERK phosphorylation, which peaked at 10 min. The addition of EGF and HRG separately, also stimulated ERK phosphorylation, which reached its climax at 15 min and gradually decreased during the subsequent 45 min. Neratinib completely inhibited the phosphorylation of ERK in all three settings.

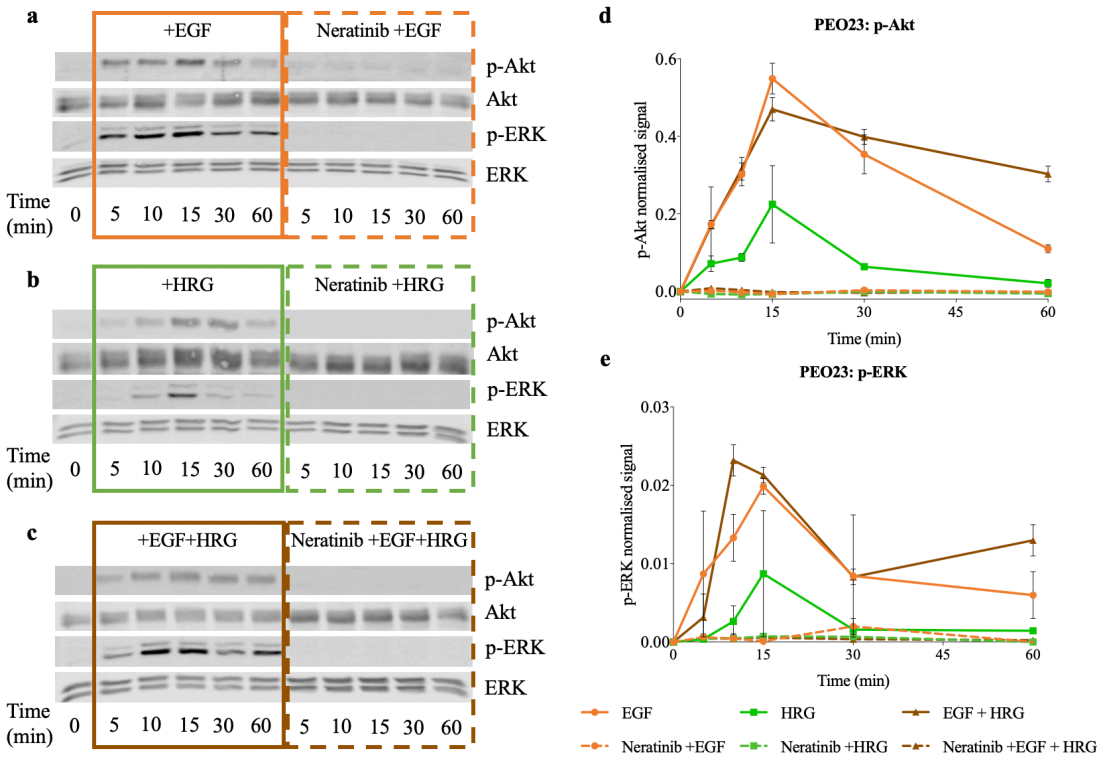


Figure 4.13: HER pathway signalling proteins for PEO23 cell line when EGF/HRG with or without neratinib was added

Western blots for (a) PEO23 with 1 ng/mL EGF stimulation ± neratinib, (b) PEO23 with 1 ng/mL HRG stimulation ± neratinib (c) PEO23 with EGF and HRG stimulation ± neratinib. Graphs representing the normalised (d) p-Akt expression with Akt from the induction of the control p-Akt (0 min), and (e) p-ERK expression with ERK from the induction of the control p-ERK (0 min). The experiment was repeated twice and the results show a representative of the results obtained for the western blots. Error bars represent standard deviation.

4.3 Effect of EGF and HRG modulation on proliferation

The effects of 1 ng/mL EGF and 1 ng/mL HRG on proliferation were assessed over a period of 120 h on all cell lines (Figure 4.14). EGF-stimulated cells grew more than the control in PEA2 and PEO1. On the other hand, HRG-stimulated cells proliferated similarly to the unstimulated controls within each cell line.

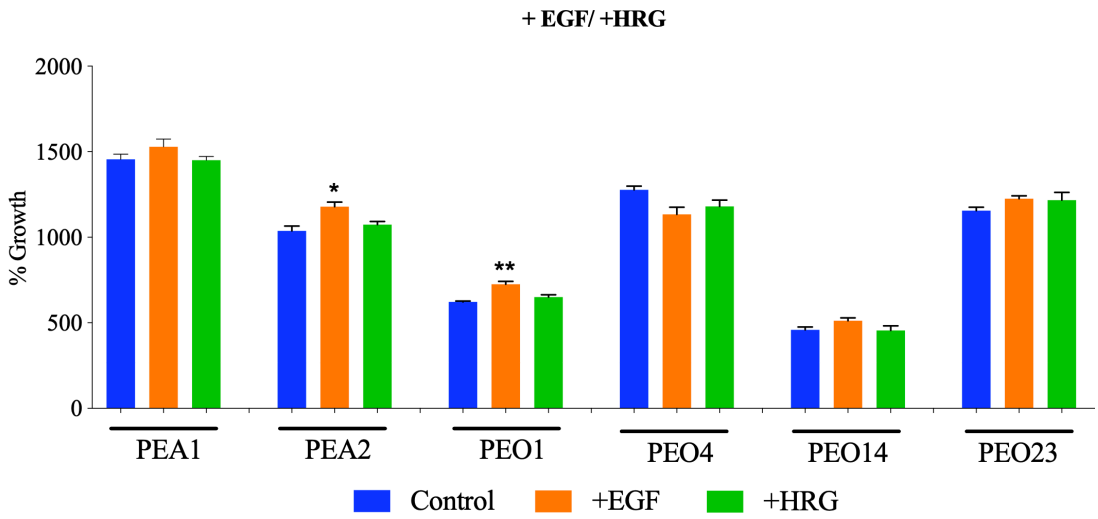


Figure 4.14: Effect of EGF and HRG on proliferation of the cell lines

*The cells were grown in charcoal stripped serum medium for 120 h, and incubated with 1 ng/mL EGF or 1 ng/mL HRG. Results are expressed as mean ± SEM of 3 technical replicates and 3 biological replicates. Statistical analysis was performed by two-way ANOVA (where *P≤0.05 and **P≤0.01).*

4.3.1 TKI influence on modulated cells

The effects of lapatinib and neratinib on the proliferation of all the cell lines in the presence of EGF and HRG was assessed using the SRB assay after 120 h simultaneous incubation. The cells were treated with a range of lapatinib and neratinib concentrations (0.003 μM – 6 μM), in the presence of 1 ng/mL EGF or 1 ng/mL HRG in charcoal stripped media. Lapatinib was chosen as a reversible inhibitor and specific to EGFR and HER2 receptors. In contrast, neratinib is an irreversible inhibitor and targets pan-HER. Additionally, neratinib was the most potent TKI of the compounds tested.

The results show that both lapatinib as well as neratinib were able to inhibit proliferation, even in the presence of EGF and HRG (Table 4.1). The cells concomitantly treated with lapatinib and EGF caused the lapatinib to be less potent against the cell lines. On the other hand, when neratinib and EGF were administered in combination, only in PEO14 did this combination cause the neratinib to be more potent within the cell line. Conversely, when lapatinib and HRG were used, PEA1, PEO1 and PEO23 cell lines were more sensitive to lapatinib treatment. PEA2, PEO1, PEO14 and PEO23 cell lines were more sensitive to neratinib when HRG was added.

	Lapatinib (μM)			Neratinib (μM)		
	Control	**EGF**	**HRG**	**Control**	**EGF**	**HRG**
PEA1	2.82	5.06****	2.03***	0.07	0.11**	0.08
PEA2	1.10	1.64**	1.31	0.06	0.09***	0.07
PEO1	0.29	0.73**	0.19	0.01	0.02	0.002
PEO4	0.54	0.98	0.63	0.05	0.10	0.07
PEO14	5.46	5.78	6.06*	0.56	0.32**	0.42****
PEO23	0.32	0.84***	0.21	0.01	0.02	0.002

Table 4.1: IC_{50} values of the cell lines treated with lapatinib and neratinib in the presence of EGF or HRG

*The green values are the ones where the IC_{50} values were below the IC_{50} value of their respective control, while the red values are the ones whose IC_{50} value was higher than the IC_{50} value of their respective control. The experiment was repeated 3 times with 3 technical replicates each time. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparisons test (where *P≤0.05, **P≤0.01, ***P≤0.001, and ****P≤0.0001).*

4.3.2 mAb influence on modulated cells

The cells were treated with 50 μg/mL pertuzumab, trastuzumab or the mAb combination (25 μg/mL + 25 μg/mL) for 120 h and incubated simultaneously with either 1 ng/mL EGF or 1 ng/mL HRG. The SRB assay was used to assess the anti-proliferative effects of these mAbs when combined with EGF and HRG.

Figure 4.15 shows the anti-proliferative effects of the mAbs when combined with EGF. The mAbs demonstrated their anti-proliferative effects even in the presence of EGF. In the PEA1, PEA2, PEO4 and PEO23 cell lines, combining EGF with all of the mAbs resulted in decreased anti-proliferative effects, especially with pertuzumab. On the other hand, in PEO1 cells, the mAbs were more potent when EGF was added. In the case of PEO14, pertuzumab had similar effects with or without EGF, trastuzumab displayed less potency with EGF and the combination of mAbs were more potent in the presence of EGF.

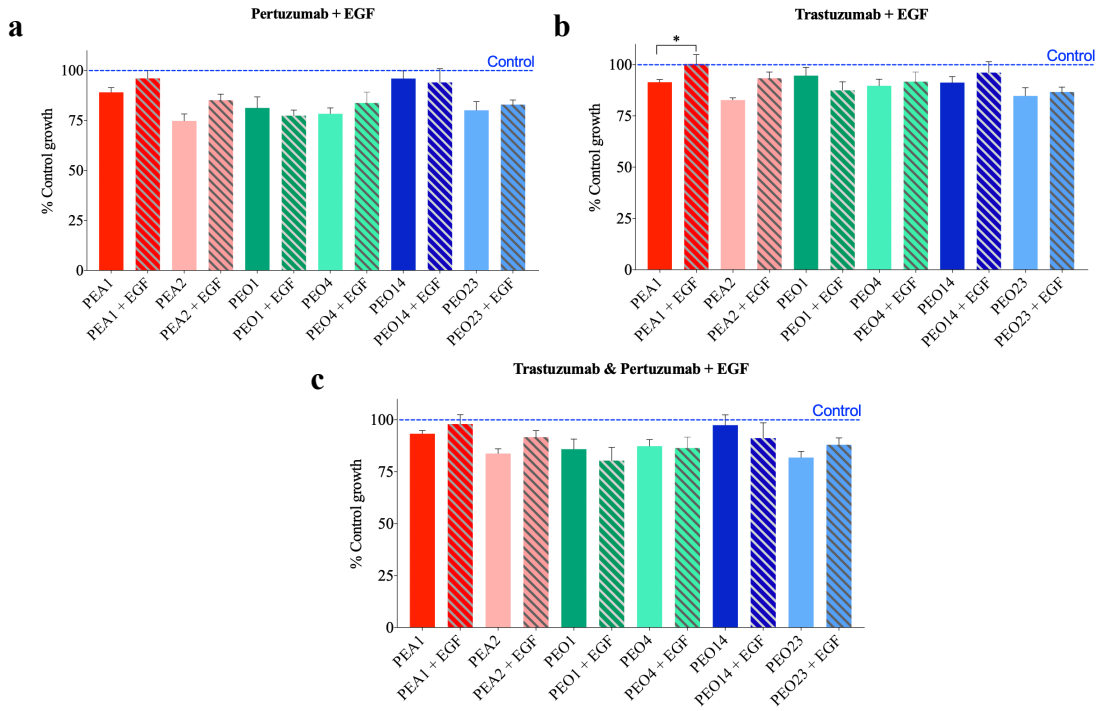


Figure 4.15: Anti-proliferative effects of mAbs when administered with EGF

*(a)50 µg/mL pertuzumab in combination with 1 ng/mL EGF, (b) 50 µg/mL trastuzumab in combination with 1 ng/mL EGF, (c) 50 µg/mL pertuzumab + trastuzumab in combination with 1 ng/mL EGF. The results show the mean percentage control growth ± SEM of 6 technical replicates and 3 biological replicates. Statistical analysis was performed by One-way ANOVA (where *P≤0.05).*

Figure 4.16 presents the graphs of anti-proliferative effects when mAbs were combined with HRG. The mAbs exhibited their anti-proliferative effects even in the presence of HRG. PEA2, PEO1, PEO4 and PEO23 cells were inhibited more effectively by the mAbs when they were combined with HRG. PEA1 and PEO14 cell lines also demonstrated improved response when HRG was added to the combination of mAbs, not to the single therapy.

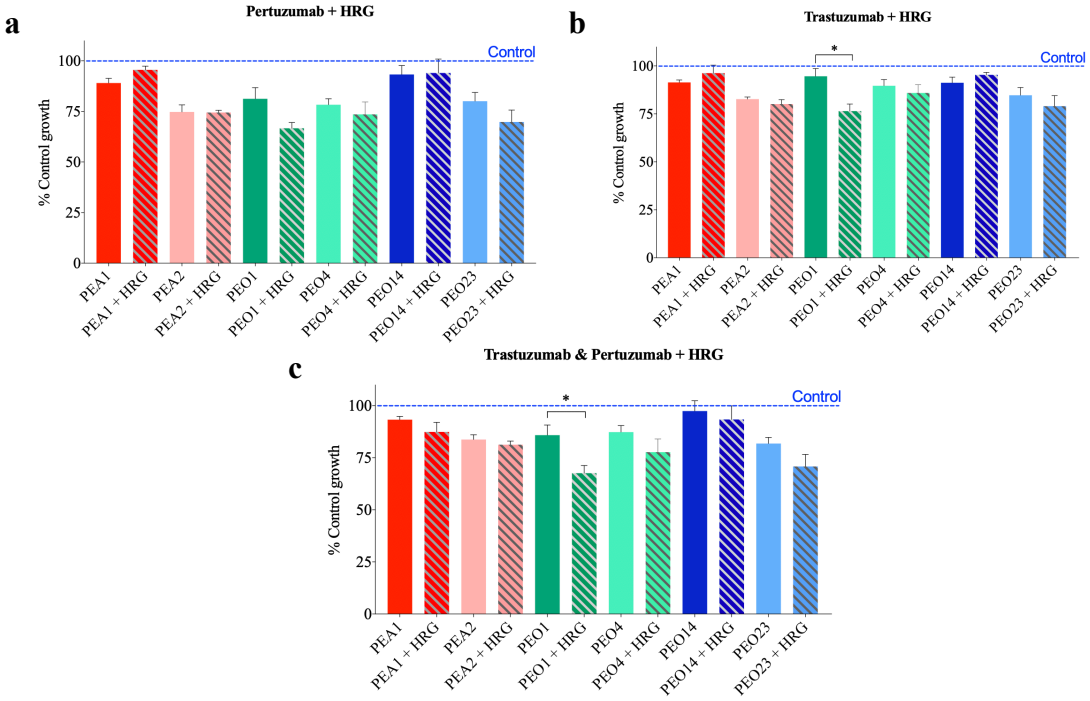


Figure 4.16: Anti-proliferative effects of mAbs when administered with HRG

*(a)50 µg/mL pertuzumab in combination with 1 ng/mL HRG, (b) 50 µg/mL trastuzumab in combination with 1 ng/mL HRG, (c) 50 µg/mL pertuzumab + trastuzumab in combination with 1 ng/mL HRG. The results show the mean percentage control growth ± SEM of 6 technical replicates and 3 biological replicates. Statistical analysis was performed by One-way ANOVA (where *P≤0.05).*

4.4 Effect of EGF and HRG modulation on migration

The PEA1-PEA2 pair of cell lines was chosen for these experiments, as the cell lines were the most migratory of those tested. Cells were seeded in 6 well plates to achieve 100% confluence the following day. A scratch was made, media changed to 0.1% FBS and 1 ng/mL EGF or 1 ng/mL HRG added to the media. Cells were allowed to migrate over 24 h. When EGF and HRG were added to the PEA1 cell line, the cells migrated more rapidly than the control (Figure 4.17). With the PEA2 cell line, the EGF-stimulated cells migrated faster than the control while the HRG-stimulated cells migrated more slowly.

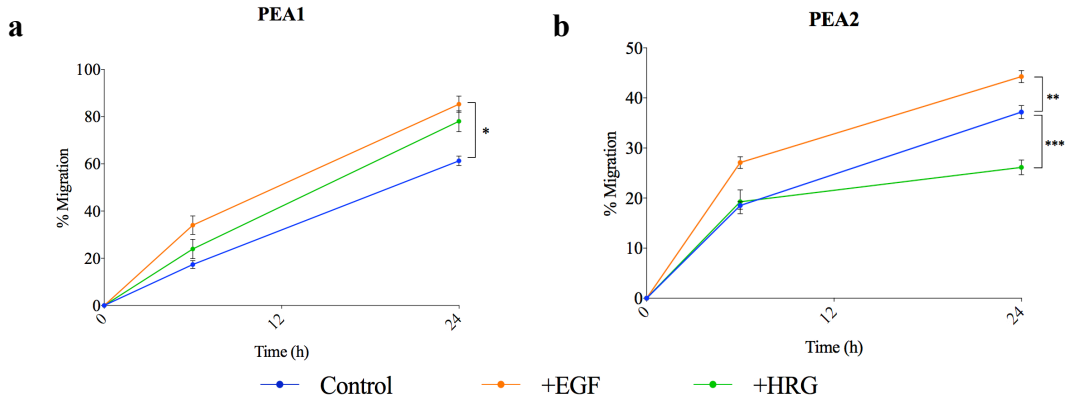


Figure 4.17: Effect of EGF and HRG on cellular migration

*(a) PEA1 cell line, (b) PEA2 cell line. Results are presented as mean ± SEM of 4 technical replicates and 3 biological replicates. Each time point is normalised to 0 h. Statistical analysis was performed by two-way ANOVA followed by Holm–Šidák test (where *P≤0.05, **P≤0.01, and ***P≤0.001).*

4.4.1 TKI influence on migration in growth factor modulated cells

To assess the anti-migratory activity of the TKIs in the presence of EGF and HRG, the scratch assay was carried out by adding each TKI (0.08 µM afatinib, 1 µM canertinib, 1.5 µM lapatinib, 0.1 µM neratinib and 1 µM sapitinib) with 1 ng/mL EGF or HRG simultaneously. The assay was performed over 24 h, and migration was assessed in relation to the 0 h scratch area.

All TKIs investigated were able to inhibit the migration of PEA1 and PEA2 cell lines even in the presence of EGF or HRG (Figure 4.18). The effects of the TKIs on EGF or HRG stimulated cells was similar to the final anti-migratory effect achieved in the absence of the growth factors. The presence of the growth factors enhanced the anti-migratory effects of the TKIs in the PEA1 cell line (Table 3.1).

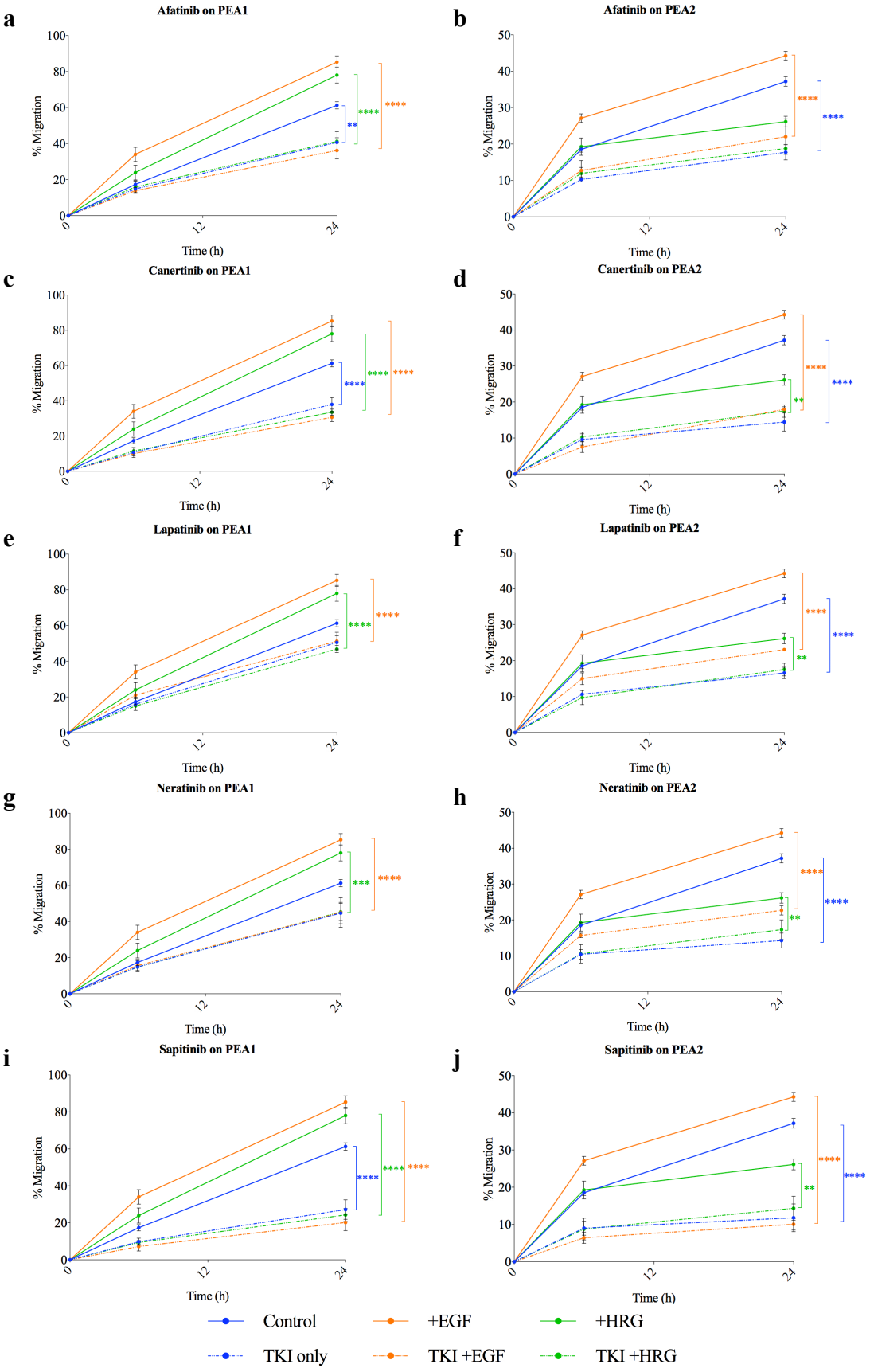


Figure 4.18: Anti-migratory effect of TKIs on EGF- and HRG- stimulated cell lines

*(a) afatinib 0.08 μM on PEA1 cell line, (b) afatinib 0.08 μM on PEA2 cell line, (c) canertinib 1 μM on PEA1 cell line, (d) canertinib 1 μM on PEA2 cell line, (e) lapatinib 1.5 μM on PEA1 cell line, (f) lapatinib 1.5 μM on PEA2 cell line, (g) neratinib 0.1 μM on PEA1 cell line, (h) neratinib 0.1 μM on PEA2 cell line, (i) sapitinib 1 μM on PEA1 cell line, and (j) sapitinib 1 μM on PEA2 cell line. Results are presented as mean ± SEM of 4 technical replicates and 3 biological replicates. Each time point is normalised to 0 h. Statistical analysis was performed by two-way ANOVA followed by Holm–Šidák test (where **P≤0.01, and ****P≤0.0001).*

	PEA1			**PEA2**		
	TKI only	+ EGF	+ HRG	TKI only	+ EGF	+ HRG
Afatinib (0.08 μM)	34%	58%	47%	52%	50%	28%
Canertinib (1 μM)	38%	64%	57%	61%	59%	33%
Lapatinib (1.5 μM)	18%	40%	40%	56%	48%	33%
Neratinib (0.1 μM)	27%	47%	42%	62%	49%	34%
Sapitinib (1 μM)	56%	76%	69%	68%	77%	45%

Table 4.2: Percentage relative inhibition on migration compared to each condition's respective control at 24 h

*Calculation: ((a-b)/a)*100; where a=% migrated by control/EGF/HRG, b= % migrated by TKI only/ TKI+EGF /TKI+HRG.*

4.4.2 mAb influence on migration in growth factor modulated cells

To assess the anti-migratory activity of the mAbs in the presence of 1 ng/mL of EGF or HRG, the scratch assay was carried out in the presence of 50 μg/mL of pertuzumab, trastuzumab or 25 μg/mL + 25 μg/mL mAb combination. The assay was done over 24 h, and migration was assessed in relation to the 0 h scratch.

In the PEA1 cell line, the addition of EGF increased the anti-migratory effect of pertuzumab, trastuzumab and the combination of mAbs in contrast to the control without growth factors (Figure 4.19, Table 4.3). The addition of HRG enhanced the anti-migratory effect in pertuzumab and the combination of mAbs with respect to the control. On the other hand, in PEA2 cell line, the addition of EGF or HRG decreased the anti-migratory capabilities of trastuzumab and the combination of mAbs, whereas the growth factors did not affect pertuzumab's ability to inhibit migration.

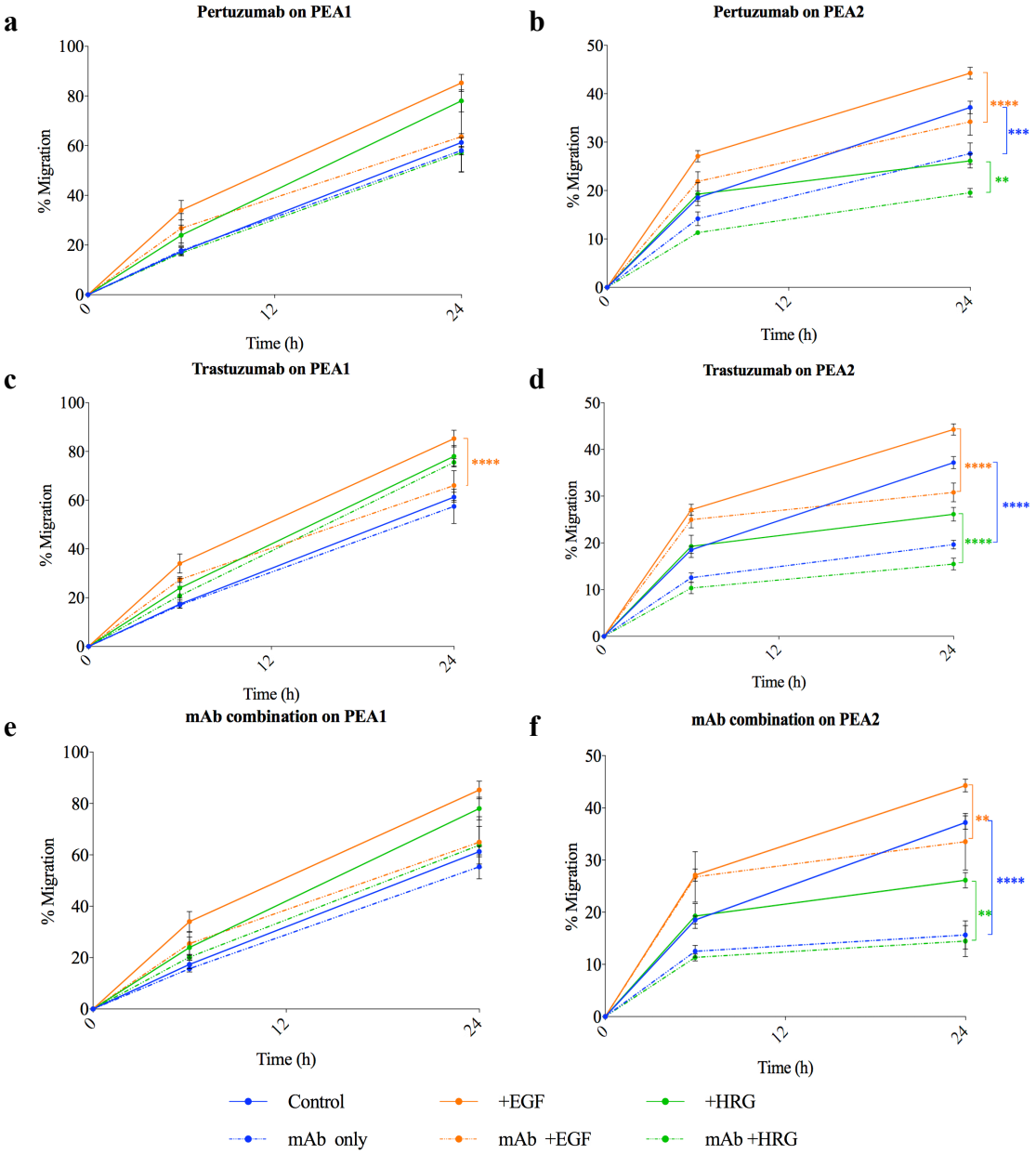


Figure 4.19: Anti-migratory effect of mAbs on EGF- and HRG- stimulated cell lines

*(a) pertuzumab 50 µg/mL on PEA1 cell line, (b) pertuzumab 50 µg/mL on PEA2 cell line, (c) trastuzumab 50 µg/mL on PEA1 cell line, (d) trastuzumab 50 µg/mL on PEA2 cell line, (e) combination 25 + 25 µg/mL on PEA1 cell line, and (f) combination 25 + 25 µg/mL on PEA2 cell line. Results are presented as mean ± SEM of 4 technical replicates and 3 biological replicates. Each time point is normalised to 0 h. Statistical analysis was performed by two-way ANOVA followed by Holm–Šidák test (where **P≤0.01, ***P≤0.001 and ****P≤0.0001).*

	PEA1			PEA2		
	mAb only	+ EGF	+ HRG	mAb only	+ EGF	+ HRG
Pertuzumab (50 µg/mL)	5%	25%	27%	26%	23%	25%
Trastuzumab (50 µg/mL)	6%	23%	3%	47%	30%	41%
Combination (25 + 25 µg/mL)	10%	24%	18%	58%	24%	45%

Table 4.3: Percentage relative inhibition on migration compared to each condition's respective control at 24 h

*Calculation: ((a-b)/a)*100; where a=% migrated by control/EGF/HRG, b= % migrated by mAb only/ mAb+EGF /mAb+HRG.*

4.5 Effect of EGF and HRG modulation on invasion

The effects of EGF and HRG in the presence or absence of neratinib in a 3D model of invasion were next investigated. This was done by positioning a spheroid per well in a 24-well plate within collagen gel, and adding 1 ng/mL EGF or HRG with or without 0.1 µM neratinib. These spheroids were allowed to invade over a period of 96 h, and the area at each time point was normalised to the area at 0 h.

4.5.1 Neratinib influence on modulated cell-spheroids

The cell line spheroids invaded more in the presence of EGF compared to the untreated control in all cell lines (Figure 4.20). Similarly, HRG increased invasion in PEO1, PEO14 and PEO23 though generally to a lesser degree than with EGF, but reduced invasion in PEA2 and PEO4 cells. Neratinib was effective at reducing invasion in all instances, irrespective of the presence of EGF and HRG. However, in the presence of EGF, neratinib was able to inhibit migration more than the control, especially in PEO1 and PEO4 cell lines, with inhibitions of 83% versus 65% in EGF-induced and controls respectively in PEO1, while in PEO4, the inhibition in EGF-induced versus control was 96% and 56% respectively (Table 4.4).

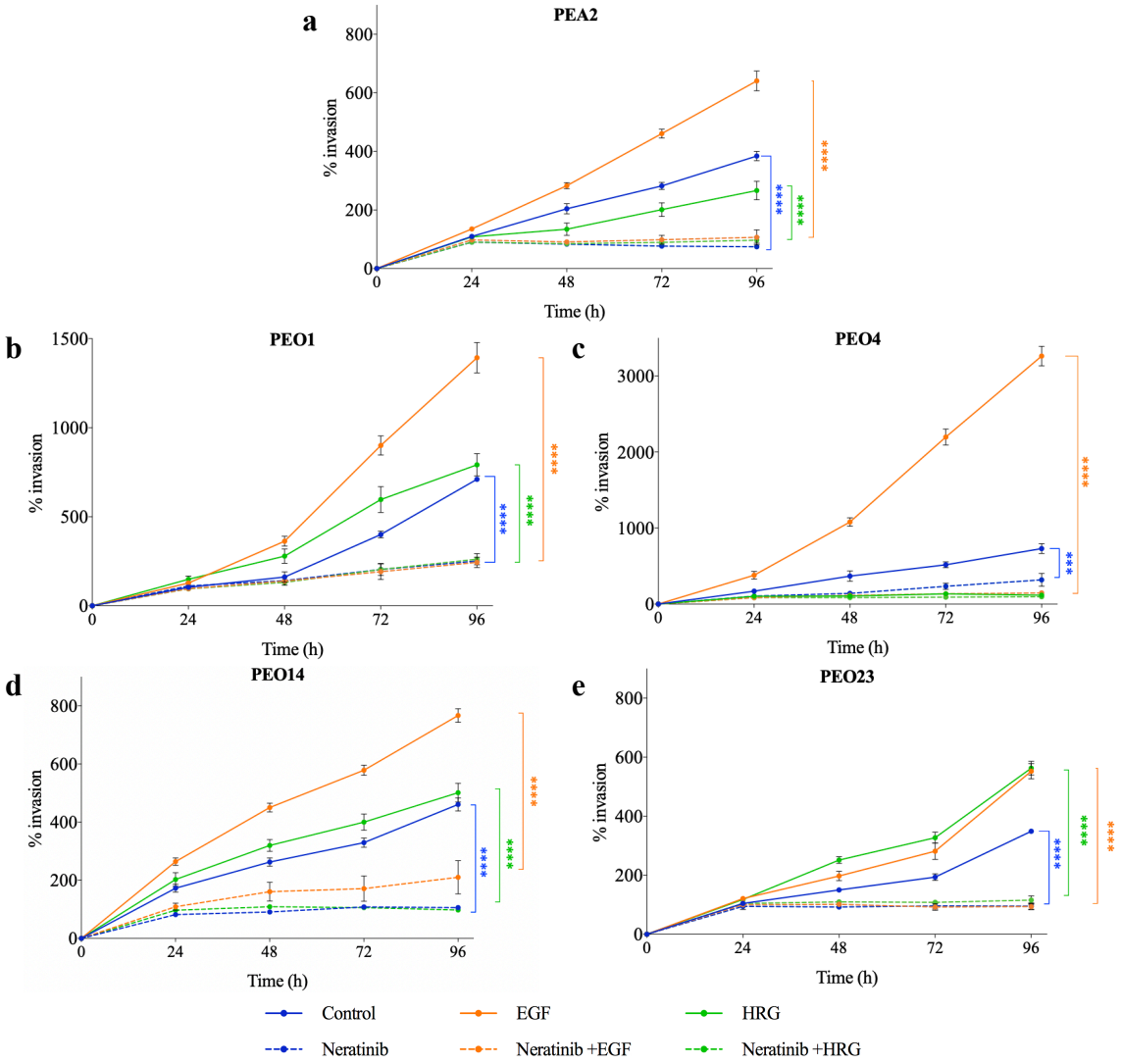


Figure 4.20: Anti-invasive effect of 0.1 μM neratinib on EGF- and HRG- stimulated cell line spheroids

*(a) PEA2, (b) PEO1, (c) PEO4, (d) PEO14, and (e) PEO23. Results are presented as mean ± SEM of at least 3 technical replicates and 3 biological replicates. Each time point is normalised to 0 h. Statistical analysis was performed by two-way ANOVA followed by Holm–Šidák test (where ***P≤0.001 and ****P≤0.0001).*

	TKI only	+ EGF	+ HRG
PEA2	80%	83%	64%
PEO1	65%	83%	67%
PEO4	56%	96%	17%
PEO14	71%	73%	81%
PEO23	71%	83%	79%

Table 4.4: Percentage relative inhibition on invasion compared to each condition's respective control at 96 h

*Calculation: ((a-b)/a)*100; where a=% migrated by control/EGF/HRG, b= % migrated by TKI only/ TKI+EGF /TKI+HRG.*

4.6 Discussion

EGF is expressed in approximately 72% of epithelial ovarian cancer tumours, and its expression was not associated with histological subtype or disease stage [406, 407]. EGF is thought to induce epithelial-mesenchymal transition in ovarian cancer, which plays an important role in cancer invasion and metastasis [408]. In ovarian cancer, approximately 90% of the tumours expressed HRG, and was found to be higher in serous than endometrioid subtypes. Additionally, HRG was not associated with disease stage or tumour grade [409]. HRG was associated with peritoneal dissemination through increased proliferation *in vitro* [410].

4.6.1 TKIs inhibit phosphorylation by EGF and HRG modulation

In this study, the effects of EGF and HRG on downstream signalling of the HER pathways were investigated. The cell lines responded differently to EGF and HRG stimulation. When EGF was added to the cell lines, this generally caused decreased expression of HER receptors, consistent with internalisation and down-regulation of EGFR. This internalisation and trafficking process to endosomes is widely reported upon ligand binding with EGFR, and has been associated with a diminished EGFR signal until EGFR levels on the cell surface are replaced [411]. On the other hand, HRG caused an overall increase in expression of the HER receptors. Generally, a reduction in EGFR expression and an increased expression of the other HER receptors, was associated with an increased phosphorylation of ERK and Akt, except in the PEO14 and PEO23 cell lines. In this case, where there was an increased EGFR expression and reduction in HER3 and HER4, an increase in phosphorylation of Akt and ERK was observed. The downstream signalling generally peaked after 48 h, which could indicate that at 24 h, many of the signalling components are being used up, and are being regenerated after 48 h.

Previous studies in ovarian cancer cell lines have suggested that EGF binds to EGFR-HER2 complexes, and also causes cross-talk between EGFR and HER3 [412]. The same study also found that activation of HER3 by HRG is independent of HER2 expression, even though HER3 preferentially binds to HER2 [412].

In section 4.1, neratinib decreased HER2 expression in the cell lines expressing HER2, consistent with studies described by Canonici *et al.* and Zhang *et al*., which have also shown that neratinib reduced HER2 expression after 12 h and 24 h in HER2+ breast cancer cell lines, possibly through moderately increasing HER2 transcription, followed by internalisation and lysosomal degradation of receptor [299, 413]. Neratinib inhibited HER4 expression in PEA2 and PEO4 cells, consistent with previous studies in breast cancer, whereby neratinib inhibited trastuzumab-induced HER4 cytoplasmic and nuclear HER4 expression [414].

All the TKIs were able to reduce the phosphorylation events investigated that were brought about by EGF and HRG stimulation. In previous studies comparing lapatinib and sapitinib's inhibitory action against HRG-stimulated phosphorylation, sapitinib produced more potent inhibition of HER2 and HER3 receptors [286]. Similarly, in this study sapitinib showed the greatest inhibitory potential overall especially in PEO1 and PEO14 cell lines, followed by neratinib on PEA2 and PEO4 cell lines and afatinib in PEO1 and PEO14 cell lines. The inhibitory potential was greater in HRG-stimulated cells, which is consistent with the fact that apart from inhibiting EGFR and HER2, sapitinib acts on HER3 receptors, whilst neratinib inhibits HER4 receptors, both of which are stimulated by HRG. Additionally, PEO1 and PEO14 cells generally had higher expressions of HER3 receptors, which might have been beneficial for the sapitinib action, whilst PEA2 and PEO4 have increased HER4 expression, required for neratinib efficacy. On the other hand, canertinib did not reduce the increased p-ERK expression brought about by HRG stimulation, especially in the PEO1, PEO4, PEO14 and PEO23 cell lines. This could be because these cells saw an increase in EGFR and a reduction in HER2 and HER4 receptors upon HRG stimulation, which could suggest there were insufficient HER2 and HER4 receptors for dimerisation. In previous ovarian cancer studies, canertinib was found to reduce p-EGFR expression but not so much p-HER2 [415], which was also observed in this study with the PEO23 cell line.

When mAbs were added to the EGF or HRG stimulated cell lines, they were only able to reduce Akt and ERK phosphorylation brought about by HRG, but not EGF. This outcome has also been described in lung cancer cell lines stimulated with EGF or HRG and inhibited by pertuzumab [416]. These findings tie well with the mode of action of

mAbs, which act specifically on HER2-containing dimers. Apart from EGFR-HER2 heterodimers, EGF can produce EGFR-based homodimerisation, which are not inhibited by these HER2 targeted mAbs. However, HRG causes HER2-HER3 heterodimerisations, which are essential for the mode of action of these mAbs, especially pertuzumab and does not result in functional HER3 homodimers due to the weak kinase activity in HER3. Additionally, Nagumo *et al.* have shown that HRG drives a lower mRNA expression of HER3 [417], which is normally associated with increased sensitivity to pertuzumab therapy [231].

From the time course experiments using phospho-specific antibodies, it was noted that the phosphorylation signals were not consistent, and sometimes were oscillating (section 4.2.3). Biological oscillations are common phenomena, and occur in circadian rhythm, cellular division cycles, and heartbeats amongst others [418]. *In vitro*, this has been observed in breast cancer cell lines, whereby addition of EGF and HRG caused oscillations in the phosphorylation of ERK and Akt. These were associated with receptor amplification, loss of transcriptional feedback and pathway crosstalk [419].

EGF and the combination of EGF and HRG had the strongest stimulation on the cell lines. Although one would hypothesise that the addition of EGF and HRG simultaneously to the cells would produce a greater receptor-specific phosphorylation effect than either growth factor alone, this was not always occurring. It might be because the ligands would antagonise each other at receptor level, or else, there were insufficient HER receptors for both ligands to bind to and produce a synergistic phosphorylation effect. Neratinib was generally effective at reducing the phosphorylation effects induced by EGF and/or HRG ligands. However, when EGF and HRG were added simultaneously, neratinib was sometimes not able to inhibit phosphorylation of the downstream signalling as effectively as with EGF or HRG alone. This might be because the extent of phosphorylation brought about by both ligands was too strong to be effectively inhibited by neratinib.

EGF, HRG or the combination produced a transient ERK activation, in the cell lines except in PEO14 cells. Transient activation is often linked with EGFR homodimers or HER3 in the absence of HER4 and has been related to cell proliferation [405]. In fact,

the PEO14 cell line had the lowest proliferation rate (section 4.3). On the other hand, prolonged activation of ERK as in the case of PEO14 is often associated with EGFR/HER2 or HER3 and HER4 activation [412]. This prolonged activation is thought to cause cell differentiation in breast cancer cell lines [420].

4.6.2 TKIs inhibit the functional effects brought about by EGF and HRG stimulation

The addition of EGF to lapatinib decreased the anti-proliferative effect of lapatinib in all six cell lines, while EGF reduced the anti-proliferative effect of neratinib in five of the six cell lines. On the other hand, treatment with HRG and lapatinib had a more potent effect than lapatinib only in three of the six cell lines, whereas four of the six cell lines were more sensitive to neratinib with HRG. This slightly contradicts results from previous studies in breast cancer, which suggested that HRG abrogated the anti-tumour effects of lapatinib, while it did not reverse the anti-proliferative effects of neratinib [421]. Another breast cancer cell line project showed that HRG-induction reduced the anti-proliferative effects of lapatinib and neratinib [422]. It could be inferred that addition of ligands could either create stronger drives which require more TKI or mAb to block the stimulation, which might be the case with EGF. Alternatively, it might produce more cellular use and dependency on the signalling pathway, thus inhibiting it might have better functional effects, as in the case of HRG.

The relative levels of expression of the HER family members may be important in determining the type of response obtained. Previous *in vitro* studies demonstrated that HRG in cell lines with high levels of HER2 compared to HER3 and HER4, produced growth inhibition. On the other hand, HRG caused stimulation in growth in cell lines with low levels of HER2 compared to HER3 and HER4 [423]. This was similar to the results encountered in this project with PEA1, PEO1, and PEO23 cells, which had a stimulatory effect in migration (PEA1) and invasion (PEO1 and PEO23) when HRG was added. These three cell lines have a low HER2 to HER3 and HER4 cell line ratio. Conversely, PEA2 and PEO4 cells had migration (PEA2) and invasion (PEA2, PEO4) inhibition on addition of HRG, but have high HER2 expression compared to HER3 and HER4.

In the cell lines that were not sensitive to TKIs, EGF and HRG induction increased the sensitivity of these cell lines towards the TKIs. For instance, EGF and HRG-modulated PEA1 cells responded better to TKIs and migration was decreased by an average of 20% in comparison to cells which did not have growth factor stimulation. Similarly, cell lines which were least responsive to neratinib's anti-invasive properties, e.g. PEO1, PEO4 and PEO23, responded more to neratinib when the cells were stimulated by EGF. On the other hand, the PEA2 cell line whose migration was originally sensitive to TKIs, did not respond better to TKIs when EGF was added to the cell line and responded worse when HRG was added to PEA2 cell line. One plausible explanation for this could be because upon the addition of EGF and HRG in PEA1 and EGF in PEA2 cells, these caused an increase in HER2 and HER3 receptors, while the addition of HRG in PEA2 caused a decrease in HER2, HER3 and HER4 receptors. That would lead to less receptors upon which the TKIs could act and produce their inhibitory effect.

When HRG was added to the mAb treatment, the mAbs still produced anti-proliferative and anti-migratory effects, sometimes even stronger than the controls. These cell lines did not express high levels of HER2 expression, which is an indication for sensitivity to trastuzumab in breast cancer [424]. Yet, previous studies in breast cancer, suggest that trastuzumab is capable of inhibiting proliferation in the presence of HRG, despite normal expression of HER2 [189, 425]. On the other hand, the addition of EGF abrogated the effects of mAbs on proliferation in all cell lines and migration in PEA2 cell lines. This links with the lack of inhibition by mAbs on EGF-induced downstream signalling of the cell lines.

In previous studies, EGF and HRG stimulation was reflected in the proliferation rates of ovarian cancer cell lines [409], however, in this study this was not as pronounced. This difference might be because previous studies have used higher concentrations of EGF and HRG (1nM, i.e. 6.4 ng/mL and 7.4 ng/mL respectively). Although MAPK signalling was increased in the cell lines when they were incubated with EGF or HRG up till 48 h (Section 4.1), this stimulation might have decreased after a while and by 120 h, would be equivalent to that in the untreated cell lines.

These experiments shed light on the importance of HER expression level, whereby differential functional effects were observed when EGF or HRG were added. This chapter shows the importance of evaluating the expression of EGF and HRG within a tumour, as the inhibitors might be less effective when certain ligands are expressed at high levels within the tumour. From these experiments, when EGF was highly expressed within a tumour, the mAbs would not produce their maximal inhibitory effect, but the presence of HRG does not reverse the inhibitory effects of mAbs as much. On the other hand, TKIs act more effectively in situations where ligands increase the availability of HER family expression, for example when HER2 and HER3 were increased, the anti-migratory and anti-invasive effects were enhanced.

5 The impact of HER expression modulation on TKI sensitivity

In Chapter 3, the results demonstrated that HER-targeted TKIs, in particular neratinib, produced anti-tumour activity in cell lines, irrespective of platinum sensitivity. The platinum-resistant cell lines generally had increased expression of HER2, HER3 or HER4, and a lower expression of EGFR. In Chapter 4, EGF and HRG activated differential HER family dimerisation, and this influenced the inhibitory effects of HER-targeted therapies, whereby for example, the presence of HRG had better effects on the inhibitory properties of neratinib, sapitinib and afatinib. Hence, this chapter aimed to look further into the role that levels of EGFR, HER2 and HER3 expression play in the HER-targeted TKI response.

Increased EGFR expression is associated with increased malignancy of ovarian cancer [136]. Additionally, it's activation by platinum therapy contributes to platinum-resistance [184]. The HER-targeted TKIs were able to inhibit phosphorylation of EGFR, as seen in Chapter 4. In this chapter, the role of 0.1 μM neratinib on cellular proliferation and migration was investigated, by knocking down EGFR, and analysing the effects that reduction of EGFR had on the PEA1 and PEA2 cell line pair. This pair was chosen as both cell lines had high EGFR expression.

HER2 overexpression or high expression is linked with increased sensitivity to HER-targeted mAbs and TKIs, especially in breast cancer [426-429]. This may also be true in ovarian cancer [130, 430], although not supported by all reports [225, 317]. On the contrary, low HER3 levels have been associated with increased response to HER2-targeted (pertuzumab) therapy [230, 231], while low HER2 expression has been associated with improved response to anti-HER3 (seribantumab) treatment [249]. Therefore, the second part of this chapter describes studies that assessed the roles of

HER2 and HER3 in the response to TKI therapy. This was done by generating stable HER2 or HER3 overexpressing cells using plasmids. In addition to the three pairs of HGSOC cell lines, the non-HGSOC SKOV3 cell line was also compared, as it is a HER2 overexpressing cell line [338].

5.1 The effect of EGFR knockdown on ovarian cancer cell lines

Transient EGFR knockdown was carried out by SiRNA transfection, using 50 nM of individual EGFR target SiRNA (as described in section 2.6 in Materials and Methods). EGFR expression was reduced by over 70% on days 2 to 5 (Figure 5.1). This knockdown was best achieved by the individual oligonucleotide J-003114-13 (J13), hence this was used in subsequent experiments. To assess proliferation, the cells were transfected 48 h after seeding and fixed 96 h after transfection. To assess migration, the cells were transfected 24 h following seeding, and the scratch was done 48 h after transfection, to allow cellular growth to reach 100% confluence. An untreated control, a mock and a 50 nM non-targeting control were also investigated, and results were normalised to untreated control and statistical analysis was compared against the non-targeting control.

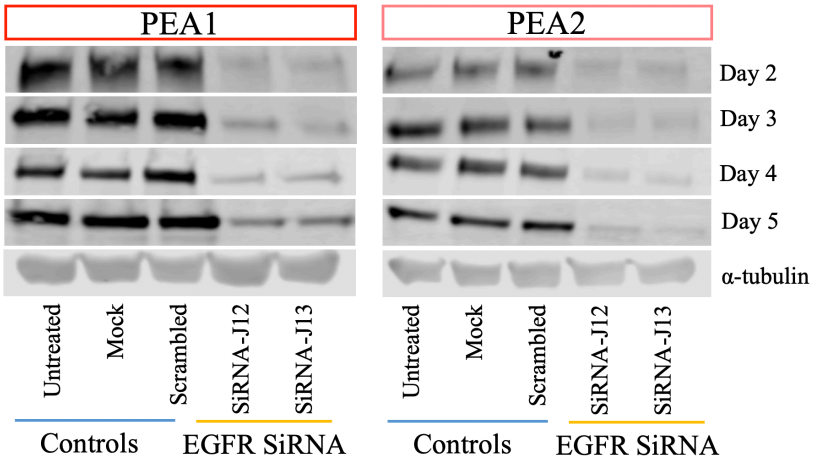


Figure 5.1: EGFR expression of EGFR-knocked down PEA1 and PEA2 cell lines

The cell lines were transfected with 50 nM of individual oligonucleotides J-003114-12 (J12) and J-003114-13 (J13). Cell lysates were collected from day 2 to day 5 after transfection to assess the level of EGFR expression. Both cell lines had their EGFR expression reduced by the targeted SiRNAs.

5.1.1 EGFR knockdown effect on proliferation

Knock-down of EGFR caused a statistically significant decrease in proliferation, especially in the platinum-sensitive cell line, PEA1 (Figure 5.2). When neratinib was added to EGFR knocked-down cells, the inhibitory effect was similar to that obtained when neratinib was added to non-EGFR knocked-down cells. However, neratinib inhibition was greater than the proliferation suppression effect occurring in EGFR knocked-down cell lines.

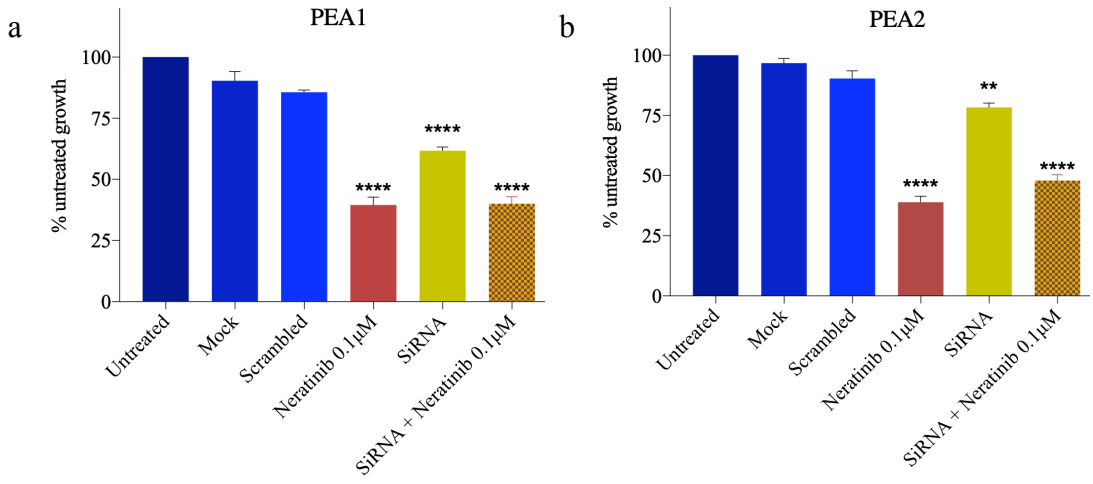


Figure 5.2: EGFR knockdown effect on neratinib anti-proliferative activity

*(a) Effect on PEA1 cell line, (b) effect on PEA2 cell line. Concentration of neratinib used was 0.1 μM, and EGFR J13 SiRNA concentration was 50 nM. Data are represented as average of 3 technical replicates and at least 4 biological replicates ± SEM. The day 0 value is subtracted from the timed value and normalised to the untreated control. One-way ANOVA was performed against the non-targeting (scrambled) control, (where **P≤0.01, and ****P<0.0001).*

5.1.2 EGFR knockdown effect on migration

When the effects of EGFR knock-down on migration were investigated, migration was decreased in cells with reduced EGFR expression (Figure 5.3). When neratinib was added to the EGFR-deficient cells, the anti-migratory effect of neratinib was not as powerful as when neratinib was added to the cells that were expressing normal EGFR levels.

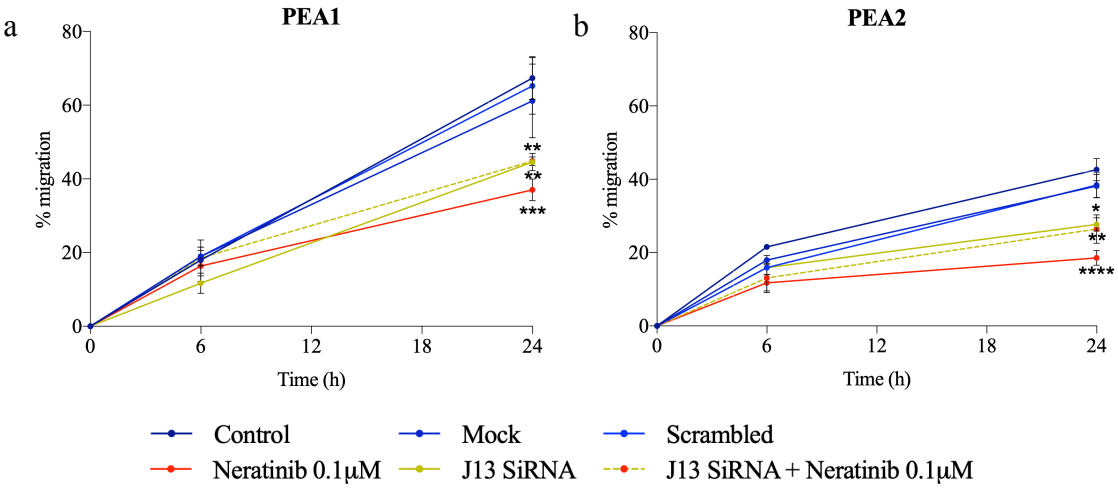


Figure 5.3: EGFR knockdown effect on neratinib anti-migratory activity

*(a) effect on PEA1 cell line, (b) effect on PEA2 cell line. Concentration of neratinib used was 0.1 µM, and EGFR J13 SiRNA concentration was at 50 nM. Data are represented as average of 5 technical replicates and at least 3 biological replicates ± SEM. The data were normalised to 0 h. Two-way ANOVA followed by Holm–Šidák test was performed against the non-targeting (scrambled) control, (where *P≤0.05, **P≤0.01, ***P≤0.001 and ****P≤0.0001).*

5.2 The effect of HER2 and HER3 overexpression on ovarian cancer cell lines

Stable transfectants with HER2 or HER3 plasmids were produced for the three pairs of WT HGSOC cell lines (PEA1-PEA2, PEO1-PEO4 and PEO14-PEO23) (as described in section 2.7 in Materials and Methods). The transfections were carried out using Lipofectamine 2000 transfection agent and individually transfected cells were selected using G418 antibiotic to prevent WT cells from contaminating the HER2 or HER3 overexpressing cells. The concentration of the antibiotic used depended on the killing curve for each WT cell line and was established before starting the transfections. The cell lines were always incubated in G418 antibiotic containing medium, to maintain selectivity of transfected cells.

5.2.1 Downstream pathway response to TKI therapy

The HER2 and HER3 transfected cell lines derived from the six cell lines (PEA1-PEA2, PEO1-PEO4 and PEO14-PEO23) were treated with the five TKIs for 24 h, and subsequently EGF or HRG were added for 30 min before lysate collection. This was done to investigate how the inhibitory activity of the HER-targeted therapy suppress pathway stimulation in the presence of EGF/HRG modulation in HER2 or HER3

overexpressing cells. Activation of the downstream products Akt and ERK were investigated to evaluate the response of these pathways to HER-targeted therapy.

In the PEA1-HER2 cell line derivative, p-HER2 expression increased in the transfected cell line, and this stimulation was inhibited by the TKIs. Expression of p-ERK and p-Akt were slightly increased in the HER2 transfected cells, and further stimulated upon EGF addition. The TKIs, reversed this stimulation. In PEA2-HER2 transfected cells, the p-HER2 expression was not affected but was inhibited by the TKIs, except sapitinib. The expression of p-ERK did not increase upon HER2 transfection, however EGF stimulation increased p-ERK expression which was reversed by lapatinib and sapitinib.

In the PEA1-HER3 cell line derivative, p-HER2 was slightly stimulated by EGF and HRG, and the signal was reversed by the TKIs. HER3 expression was unaffected by the TKIs. Expression of p-ERK was stimulated in the cell line when EGF was added, which was reversed by all TKIs, whereas addition of HRG caused stimulation of p-Akt expression, which was reversed by afatinib, lapatinib, neratinib and sapitinib. In the HER3 transfected PEA2 cell line, p-HER2 was unchanged with the addition of EGF or HRG, but was inhibited by the TKIs. Addition of EGF increased p-ERK expression, and this increased phosphorylation was reduced by all five TKIs under investigation.

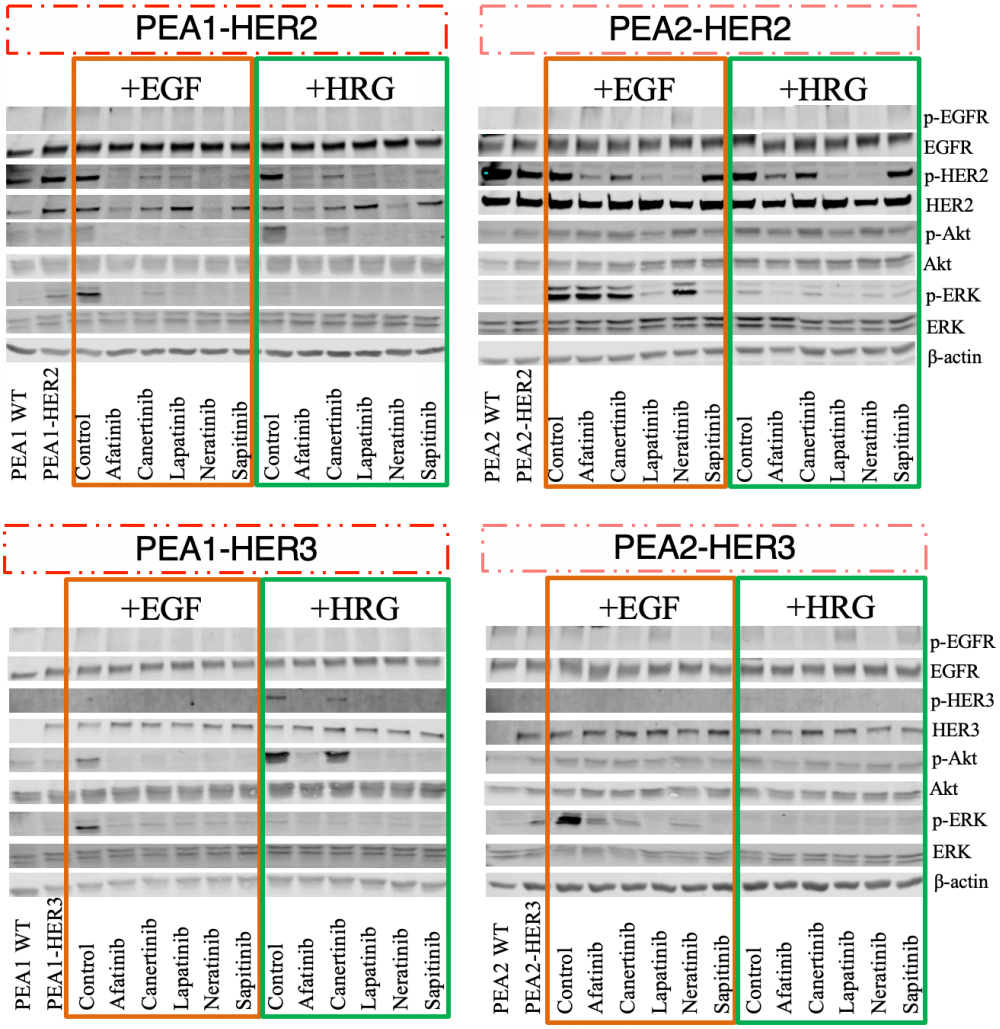


Figure 5.4: HER pathway and downstream signalling proteins for PEA1 HER2 or HER3 and PEA2 HER2 or HER3 modulated cell lines when EGF/HRG with or without TKIs were added

TKIs were added at clinically relevant concentrations for 24 h; 30 min before lysate collection, 1 ng/mL EGF or HRG were added to the cells. The experiment was repeated once and the loading controls are a representation to the other membranes.

The other four cell lines PEO1-PEO4 and PEO14-PEO23 were also investigated, and they produced similar results to PEA1-PEA2 cell line pair (see Appendix 1).

5.2.2 Proliferation response to TKI therapy

The effects of the five TKIs and the cytotoxic drugs (cisplatin, carboplatin and paclitaxel) in high HER2 and HER3 expressing cell line transfects were investigated to evaluate their properties on sensitivity to therapy. The cells were incubated with the TKIs or the cytotoxic agents for 120 h in 10% FCS serum conditions, without growth ligands.

The TKIs and cytotoxic agents inhibited the proliferation of the cell lines in a concentration-dependent manner (Figure 5.5). The HER2 and HER3 transfected PEA1 cell line derivatives were, in general, more sensitive to the TKIs than the parental cell line (Table 5.1). On the contrary, the HER2 or HER3 transfected PEA2 cell lines were more resistant to the TKIs than the PEA2 WT cell line. Similar effects were noticed with the platinum-based chemotherapy. However, the PEA1 cell line derivatives were more resistant to paclitaxel than the parental PEA1 cell line, while PEA2 cell line derivatives were more sensitive to paclitaxel.

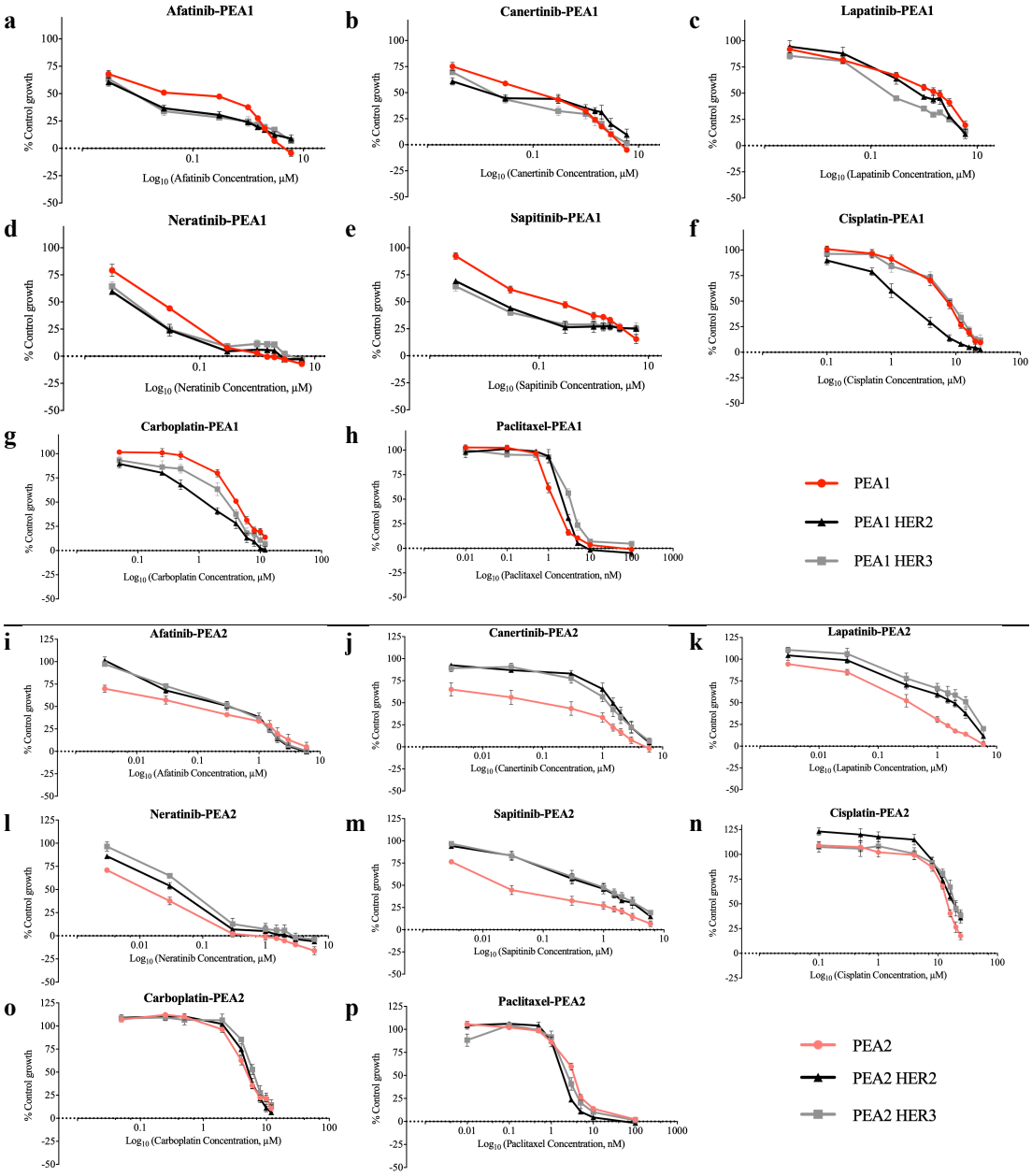


Figure 5.5: Concentration-response curves for PEA1/PEA2 HER2/HER3 cell derivatives

(a-h) Effects of TKIs and cytotoxic agents on PEA1 cell line and its HER2 or HER3 derivatives after 120 h (a) afatinib, (b) canertinib, (c) lapatinib, (d) neratinib, (e) sapitinib, (f) cisplatin, (g) carboplatin, and (h) paclitaxel. (i-p) Effects of TKIs and cytotoxic agents on PEA2 cell line and its HER2 or HER3 derivatives after 120 h (i) afatinib, (j) canertinib, (k) lapatinib, (l) neratinib, (m) sapitinib, (n) cisplatin, (o) carboplatin, and (p) paclitaxel. The results show the mean percentage control growth ± SEM of 6 technical replicates and 3 biological replicates.

IC_{50} values	**PEA1**			**PEA2**		
	Control	**HER2**	**HER3**	**Control**	**HER2**	**HER3**
Afatinib (μM)	0.35	<0.01***	0.01**	0.32	0.54	0.55
Canertinib (μM)	0.38	0.10	0.10	0.28	1.23****	1.53****
Lapatinib (μM)	1.65	1.17	0.27****	0.45	2.30****	1.67****
Neratinib (μM)	0.02	0.01	0.01	0.02	0.05***	0.04***
Sapitinib (μM)	0.23	0.02****	0.02****	0.02	0.87****	0.83****
Cisplatin (μM)	7.18	1.93****	8.05	14.65	19.70***	17.98****
Carboplatin (μM)	4.13	1.65****	2.90****	4.89	6.18*	5.38
Paclitaxel (nM)	1.49	2.51**	3.36****	3.40	2.77*	2.15***

Table 5.1: IC_{50} values of HER2 or HER3 modulated PEA1 and PEA2 cell line pair

*Concentrations of the TKIs tested ranged between 0.003-6 μM, cisplatin tested varied between 0.05-12 μM, carboplatin tested ranged between 0.1-24 μM, and paclitaxel ranged between 0.01-100 nM incubated for 120 h. Data are represented as IC_{50} values calculated in GraphPad Prism, based on an average of 6 technical replicates and at least 3 biological replicates. The day 0 value is subtracted from the timed value and normalised to the vehicle control. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparisons test (where *P≤0.05, **P≤0.01, ***P≤0.001, and ****P≤0.0001). The green boxes signify increased sensitivity, red boxes represent decreased sensitivity as opposed to the respective WT cell line.*

The PEO1 HER3-transfected cells were more sensitive to TKI and cytotoxic therapy than the PEO1 parental cell line (Table 5.2). Similarly, the HER2-overexpressing PEO1 cell line was more sensitive to the TKI and cytotoxic therapy than the parental cell line, except with canertinib, cisplatin and carboplatin therapy. On the contrary, the HER2 and HER3 transfected PEO4 cell lines were more resistant to the TKIs and cytotoxic agents than the PEO4 parental cell line, except for carboplatin which was more potent in the transfected cells.

IC_{50} values	PEO1			PEO4		
	Control	HER2	HER3	Control	HER2	HER3
Afatinib (μM)	0.39	0.18****	<0.10**	0.68	0.92*	0.69
Canertinib (μM)	0.60	0.73	<0.10****	0.96	1.63****	1.61****
Lapatinib (μM)	1.95	1.12****	0.22****	1.64	3.27****	2.80****
Neratinib (μM)	0.03	0.02***	0.01****	0.04	0.08*	0.04
Sapitinib (μM)	0.40	0.26**	0.02****	0.34	1.37**	1.68***
Cisplatin (μM)	3.89	8.13****	3.46*	13.33	18.3****	16.6****
Carboplatin (μM)	1.68	3.71****	1.55***	5.20	4.07***	5.14
Paclitaxel (nM)	2.58	2.34	1.81*	2.41	3.50***	2.49

Table 5.2: IC_{50} values of HER2 or HER3 modulated PEO1 and PEO4 cell line pair

*Concentrations of the TKIs tested ranged between 0.003-6 μM, cisplatin tested varied between 0.05-12 μM, carboplatin tested ranged between 0.1-24 μM, and paclitaxel ranged between 0.01-100 nM incubated for 120 h. Data are represented as IC_{50} values calculated in GraphPad Prism, based on an average of 6 technical replicates and at least 3 biological replicates. The day 0 value is subtracted from the timed value and normalised to the vehicle control. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparisons test (where *P≤0.05, **P≤0.01, ***P≤0.001, and ****P≤0.0001). The green boxes signify increased sensitivity, red boxes represent decreased sensitivity, and orange boxes represent equal sensitivity as opposed to the respective WT cell line.*

The PEO14 HER2 and HER3 cell line derivatives were more sensitive to the TKIs than their parental cell line, with the HER3 derivatives being more sensitive than the HER2 derivatives (Table 5.3). Both derivatives were more resistant to cisplatin therapy while only HER3 derivative was more resistant to carboplatin therapy. On the other hand, the PEO23 cell line HER2 or HER3 derivatives were more resistant to the TKIs and cytotoxic therapy. The exceptions were sapitinib and paclitaxel, where the HER2 derivative was more sensitive to these inhibitors than the parental cell line.

IC_{50} values	PEO14			PEO23		
	Control	HER2	HER3	Control	HER2	HER3
Afatinib (μM)	1.68	1.21***	0.64****	0.47	0.67	0.78**
Canertinib (μM)	2.44	1.80****	1.58***	0.84	1.91****	2.07****
Lapatinib (μM)	6.09	4.32****	2.56****	2.21	3.24****	2.87**
Neratinib (μM)	1.05	0.42****	0.03****	0.02	0.09***	0.06*
Sapitinib (μM)	7.11	4.03****	1.20****	1.97	1.55	1.92
Cisplatin (μM)	4.93	5.74**	15.1****	13.98	21.1****	19.3***
Carboplatin (μM)	3.76	2.60****	4.19*	5.19	7.02****	5.29*
Paclitaxel (nM)	2.15	1.17*	1.97	2.42	2.05	2.84

Table 5.3: IC_{50} values of HER2 or HER3 modulated PEO14 and PEO23 cell line pair

Concentrations of the TKIs tested ranged between 0.003-6 μM, cisplatin tested varied between 0.05-12 μM, carboplatin tested ranged between 0.1-24 μM, and paclitaxel ranged between

*0.01-100 nM incubated for 120 h. Data are represented as IC_{50} values calculated in GraphPad Prism, based on an average of 6 technical replicates and at least 3 biological replicates. The day 0 value is subtracted from the timed value and normalised to the vehicle control. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparisons test (where *P≤0.05, **P≤0.01, ***P≤0.001, and ****P≤0.0001). The green boxes signify increased sensitivity, red boxes represent decreased sensitivity, and orange boxes represent equal sensitivity as opposed to the respective WT cell line.*

The effects of TKIs on the HER2-overexpressing SKOV3 non-HGSOC cell line were also investigated to compare with the HER2-overexpressing HGSOC cell lines (Figure 5.6a). The TKIs inhibited the SKOV3 cell line in a concentration-dependent manner. The IC_{50} values were higher compared to the transfected HER2 overexpressing HGSOC cell lines (Figure 5.6b, Table 5.4). SKOV3 was resistant to lapatinib, with IC_{50} values of over 6 μM, whilst the most potent TKI was neratinib with IC_{50} value of 0.2 μM.

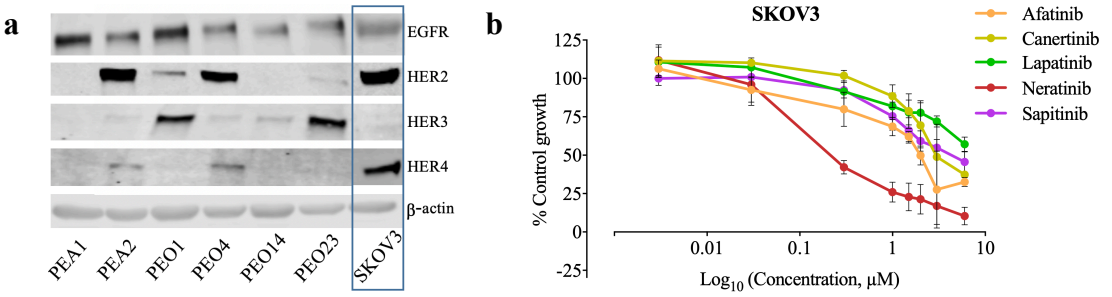


Figure 5.6: SKOV3 cell line characteristics and anti-proliferative effects of TKIs

(a) Protein expression of the 4 HER receptors in SKOV3 cell line was detected by western blot. (b) Concentration-response curves of the TKIs on SKOV3 cell line after 120 h. The results show the mean percentage control growth ± SEM of 6 technical replicates and 3 biological replicates.

IC_{50} values	SKOV3
Afatinib (μM)	1.84
Canertinib (μM)	3.09
Lapatinib (μM)	>6.00
Neratinib (μM)	0.23
Sapitinib (μM)	3.80

Table 5.4: IC_{50} values of TKIs in SKOV3 cell line

Concentrations of the TKIs tested ranged between 0.003-6 μM. Data are represented as IC_{50} values calculated in GraphPad Prism, based on an average of 6 technical replicates and 3 biological replicates. The day 0 value is subtracted from the timed value and normalised to the vehicle control.

5.2.3 Migratory response to TKI therapy

The anti-migratory abilities of TKIs in cell lines expressing increased levels of HER2 or HER3 were investigated. In the PEO1 cell line, enhanced expression of HER2 or HER3 increased the migratory ability of the cells, especially with HER2 (Figure 5.7a). The TKIs reduced migration in both HER2 and HER3 transfected cell lines. The anti-migratory effect of TKIs was stronger in the HER3 transfected cells, with canertinib, lapatinib and neratinib producing the greatest inhibition (Figure 5.7b, c, and Table 5.5).

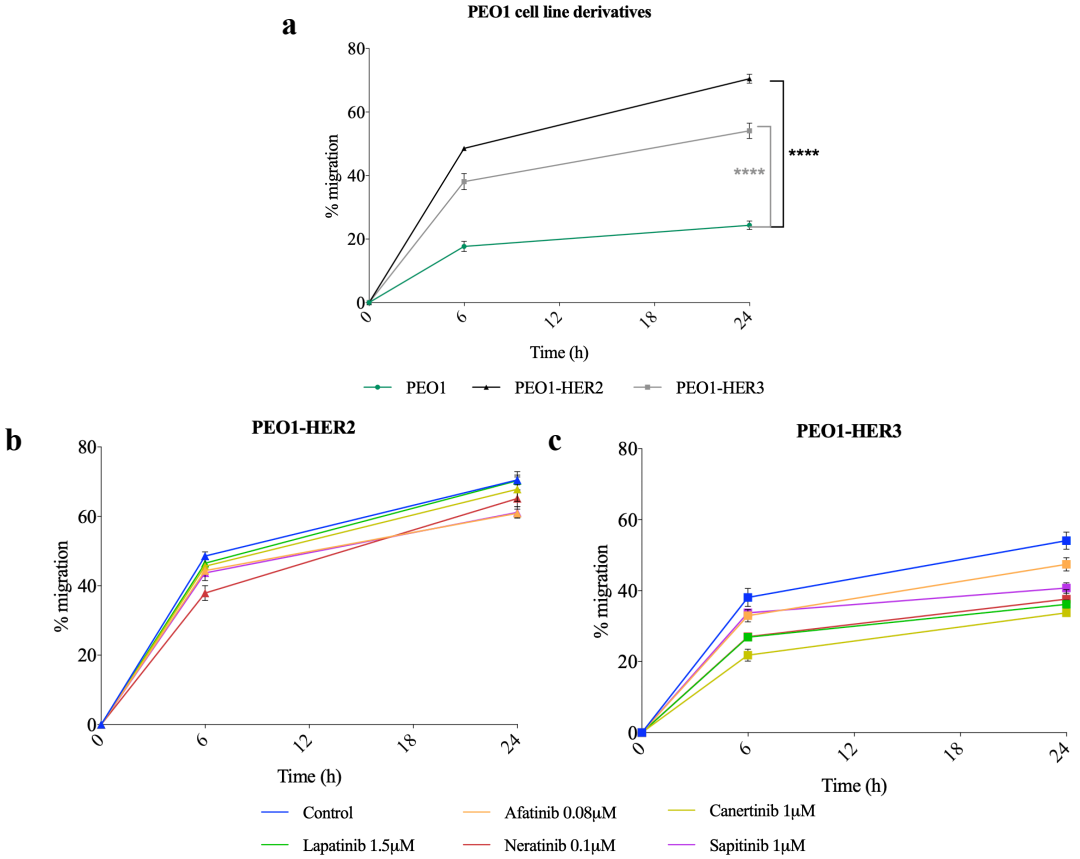


Figure 5.7: Effects of TKIs on PEO1 HER2 or HER3 cell line derivatives

*The graphs show the migratory properties of PEO1 cell line transfects in a 24 h period. (a) PEO1 cell line transfects migration properties, (b)TKIs on PEO1-HER2 cell line, (c) TKIs on PEO1-HER3 cell line. Data are represented as average of 6 technical replicates and at least 4 biological replicates ± SEM. The data are normalised to 0 h. Statistical analysis was conducted using two-way ANOVA (where **** P≤0.0001).*

	PEO1-HER2	PEO1-HER3
Afatinib (0.08 µM)	13.6%	12.3%
Canertinib (1 µM)	3.8%	37.6%
Lapatinib (1.5 µM)	0.2%	33.1%
Neratinib (0.1 µM)	7.6%	30.4%
Sapitinib (1 µM)	13.2%	24.6%

Table 5.5: Percentage relative inhibition of migration in PEO1 cell line derivatives compared to control at 24 h

*Calculation: ((a-b)/a)*100; where a=% migrated by control, b= % migrated by TKI, as explained in section 2.2.2.*

The anti-migratory effects of TKIs on the SKOV3 cell line were investigated to compare their effects on non-HGSOC versus HGSOC HER2 high expressing cell lines. All TKIs inhibited migration of the SKOV3 cell line, with sapitinib and afatinib having the strongest anti-migratory effects (Figure 5.8, Table 5.6).

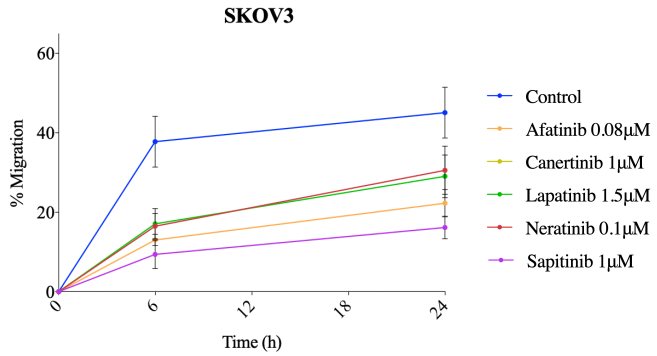


Figure 5.8: Effects of TKIs on SKOV3 cell line migration

The graph shows the migratory properties of SKOV3 cell line in a 24 h period in response to TKIs. Data are represented as average of 6 technical replicates and 3 biological replicates ± SEM. Statistical analysis was performed by two-way ANOVA. The data are normalised to 0 h.

	SKOV3
Afatinib (0.08 µM)	51%
Canertinib (1 µM)	42%
Lapatinib (1.5 µM)	36%
Neratinib (0.1 µM)	32%
Sapitinib (1 µM)	64%

Table 5.6: Percentage relative inhibition on migration in SKOV3 cell line compared to control at 24 h

*Calculation: ((a-b)/a)*100; where a=% migrated by control, b= % migrated by TKI, as explained in section 2.2.2.*

5.3 Discussion

Multiple studies have shown the importance and potential biomarker use of HER expression in the HER-targeted TKI response [305, 431-435]. In the case of HER2, there are contradicting studies as to the level of expression needed for optimum HER-targeted antibody therapy response in ovarian cancer cells. Some studies using xenograft experimental models suggest that high HER2 (amplified) expression is necessary at least in a non-HGSOC setting for trastuzumab/pertuzumab anti-tumour activity [232], while others suggest that therapy can work even at low HER2 expression levels for trastuzumab [316, 317] and also for the anti-HER3 antibody seribantumab [249]. Low HER3 expression has been associated with response to anti-HER2 pertuzumab [230, 231], while for EGFR, mutational status may be important as suggested by the isolated response to gefitinib in a phase II ovarian clinical trial [267]. This chapter aimed to further investigate the role of EGFR, HER2 and HER3 expression levels in response to TKI treatment.

5.3.1 Impact of EGFR expression on proliferation and migration

Activation of EGFR is associated with increased proliferation and migration, in experimental models of ovarian cancer [436-438]. In the present study, when EGFR was knocked down, it produced a more significant decrease in proliferation in PEA1 cells than in PEA2 cells. EGFR knock-down also resulted in decreased migration of both cell lines. The anti-proliferative and anti-migratory response of neratinib in EGFR knocked-down cells was assessed to help evaluate the importance of EGFR expression level in this regard. Neratinib reduced the proliferation of PEA1 and PEA2 cell lines further than the proliferation of EGFR-depleted cells. This suggests that while EGFR is playing an important role in cellular proliferation, neratinib depends not only on EGFR to elicit its anti-proliferative effects, and is therefore likely inhibiting other HER receptors, consistent with its broad spectrum activity. Previous studies have also shown that the anti-proliferative effects of neratinib correlate with HER2 baseline expression but not EGFR expression in breast cancer cell lines [299]. This EGFR-independent anti-proliferative effect was also noticed when lapatinib was used in EGFR depleted, HER2+ breast cancer cell lines, where EGFR knockdown did not reduce the potency of lapatinib [439] or HER-targeted mAbs [440] and when gefitinib

was added to HER2 overexpressing, EGFR deficient lung cancer cells [441]. On the contrary, although EGFR knockdown decreased the migratory ability of PEA1 and PEA2 cell lines, neratinib did not decrease this migration further. This is consistent with neratinib requiring EGFR for its anti-migratory effects.

5.3.2 Impact of HER2 and HER3 overexpression on proliferation and migration

The TKIs decreased the p-HER2 expression that was stimulated by EGF or HRG in the HER2-overexpressing PEA1 cell line. Lapatinib and sapitinib inhibited the p-ERK expression increase brought about by EGF, while all TKIs reversed HRG-stimulated p-ERK. Stimulation of p-ERK with EGF or HRG in the HER3-overexpressing cell line was reversed by all TKIs.

In the platinum-sensitive cell lines, increased expression of HER2 or HER3 led to increased sensitivity to the TKIs. On the contrary, higher HER2 or HER3 expression in the platinum-resistant cell lines led to more resistance to the TKIs. HER2 overexpression has been linked with increased response to paclitaxel in breast cancer [429], however this was not always observed in the HGSOC HER2 overexpressing cell lines investigated in this chapter. These results suggest that although more HER expression increases sensitivity to inhibitors, there is a limit to the receptor occupancy. Overly high expression levels might not be as favourable for optimum response to therapy in HGSOC, as the maximum occupancy level of the receptors would be reached. Other studies also suggested that the ratio of HER2 to HER3 (and not HER2:HER4) plays an important role for proliferation control [423] and HER-targeted therapy response. Additionally, another study has found that inhibition of HER3 provides better therapeutic potential than inhibition of EGFR in HER2 positive breast cancer [440]. That study also found that knocking down HER3 decreases the proliferation to the same extent as HER2 knockdown. Moreover, p-HER3 was often detected when HER2 was overexpressed [440], which sheds light about the importance of HER2-HER3 complex as an oncogene [442]. In breast cancer, the levels of ligands such as HRG, are also important for the proliferation control, as HRG mediates HER2-HER3 heterodimers [443].

In breast cancer and lung cancer clinical trials, HER2+ patients respond better to afatinib, lapatinib, neratinib [287, 299, 435, 444, 445]. However, in the case of the SKOV3 cell line, increased HER2 expression did not correlate well with better anti-proliferative response to TKIs, especially with lapatinib. The IC_{50} values achieved are all higher than the concentrations achieved in human serum after appropriate dosing. On the other hand, the anti-migratory response of the TKIs on the SKOV3 cell line was approximately 4-fold better in comparison to other HER2 positive cell lines and the HER2-overexpressing PEO1 cell line. These seemingly contradictory results of the SKOV3 cell line compared against HER2 overexpressing HGSOC cell lines suggest that non-HGSOC cells respond differently to TKIs, and necessitate separate evaluation.

This chapter demonstrated that HER expression levels can play an important role in the response to HER-targeted therapy. The HER2 and HER3 expression appear to be important for HGSOC response to therapy, whereas EGFR expression is less important for response to therapy. Additionally, non-HGSOC tumours cannot be directly compared with HGSOC tumours for their response to therapy, and should be studied separately.

6 Gene expression response to HER-targeted therapy and growth factors

In the previous chapters, neratinib was shown to demonstrate anti-proliferative, anti-migratory and anti-invasive properties across the panel of platinum-sensitive and platinum-resistant HGSOC cell lines, even when cell lines were stimulated by EGF or HRG. In the first part of this chapter, experiments are described where the aim was to analyse the genes that are differentially expressed between the platinum-sensitive (PEA1) and platinum-resistant (PEA2) ovarian cancer cell lines, to evaluate the genes induced by HRG stimulation and which pathways are affected by ligand stimulation, and to identify differences between the non-treated or HRG-treated and neratinib treated cells to compare which pathways are affected by neratinib and other HER-targeted inhibitors. In the second part of the chapter, genes of interest that were differently expressed upon neratinib treatment were identified and their proteins were assessed in previously constructed TMAs that contained SKOV3 tumour xenografts treated with the HER-targeted mAbs. The experiments had been carried out *in vivo*, and the mice had been treated with pertuzumab (20 mg/kg), trastuzumab (20 mg/kg) or the combination (20 mg/kg each) and samples were collected on days 1, 2, 4, 7, and 14 [232, 233].

RNAseq gene expression analysis was used to study the transcriptomes of PEA1 and PEA2 cells in the presence of HRG and/or neratinib (quality control data for the samples can be found in Appendix 2). Four treatment groups were compared, and three replicates for each group were prepared (Figure 6.1). HRG was chosen over EGF as a previous study investigating the role of EGF or HRG stimulation in breast cancer cell lines demonstrated that the activation by both ligands resulted in similar differently

expressed genes, however, HRG resulted in more differently expressed genes than EGF [405].

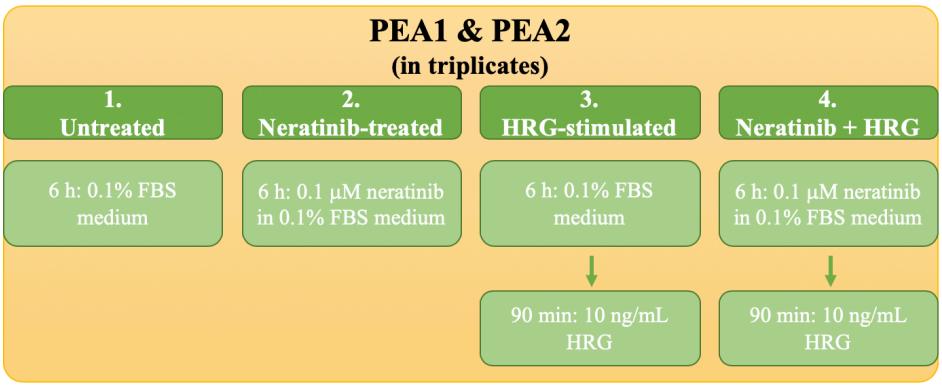


Figure 6.1: Overview of gene expression analysis treatment groups
There were 4 different groups for each cell line, which included the untreated controls, neratinib-treated (treated for 6 h with 0.1 μM neratinib), HRG-stimulated (0.1% serum, deprived for 6 h, and 10 ng/mL HRG added for 90 min), neratinib + HRG-treated (treated for 6 h with neratinib, and 10 ng/mL HRG added for 90 min).

6.1 Differential gene expression: platinum-sensitive versus platinum-resistant cell lines

The gene expression differences between the platinum-sensitive PEA1 cell line and the platinum-resistant cell line PEA2 gathered from the RNAseq data were analysed using the Rank Product function within TMeV, with the false discovery rate (FDR) being set at 5%. This was done to evaluate the main differences between platinum-sensitive and platinum-resistant HGSOC and investigate the correlation between gene expression and what was observed *in vitro*.

RNAseq yielded 16,352 gene expressions values. There were 1,387 (about 8% of the gene yield) more highly expressed genes and 1,368 (about 8% of the gene output) more lowly expressed genes in the PEA2 cell line versus the PEA1 cell line. The DAVID online resource[5] revealed the pathways that were more highly expressed in the PEA1 cell line included the ECM-related, focal adhesion and PI3K-Akt pathways (Figure

[5] DAVID online resource available from: https://david.ncifcrf.gov.

6.2a). The pathways that were more highly expressed in the PEA2 than in the PEA1 cell line included the Wnt signalling and MAPK pathways.

Clustering the highlighted biological processes between the cell lines (Figure 6.2b) demonstrated that genes most highly expressed in the PEA1 cell line included genes associated with cell cycle (example *CDKN2A* and *MYC*) and cell migration processes (such as *MET* and *ENG*). Genes that were more highly expressed in the PEA2 cell line than in PEA1 cells included genes associated with cell adhesion, tyrosine kinase activity (including *HER2*, *HER3* and *HER4*) and Wnt (wingless) activity.

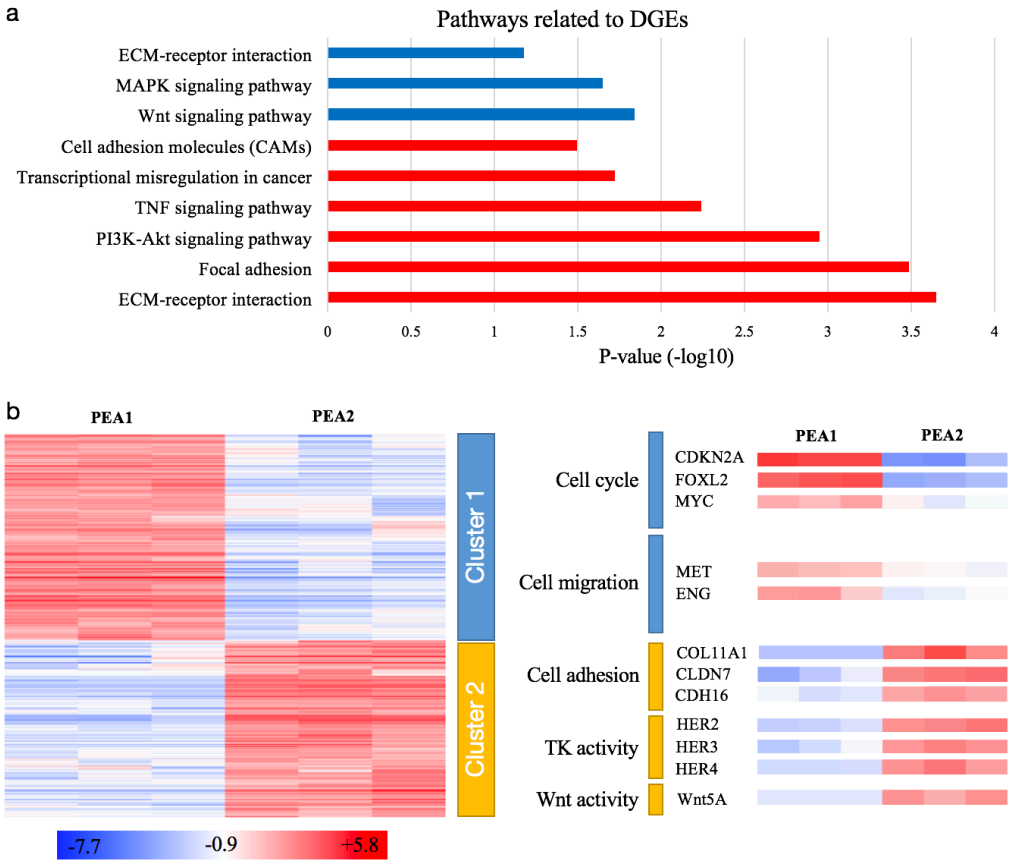


Figure 6.2: Analysis of differently expressed genes between the PEA1 and PEA2 cell line pair

(a) Pathways related to the differently expressed genes and the –log10 p-value of each pathway analysed using DAVID online resource. The blue bars represent the pathways that are lower expressed in PEA1 cell line with respect to PEA2 cell line, while the red bars represent the pathways that are more highly expressed in PEA1 cell lines versus PEA2 cell line. (b) Log2 mean centred heatmap on the differently expressed genes between PEA1 and PEA2 cell line pair. Red represents higher relative expression and blue lower relative expression. The genes were clustered and average linkage was applied in Cluster 3.0, and the heatmap was drawn in Treeview. The genes in each cluster were analysed for their biological processes in DAVID online resource.

6.2 Differential gene expression: untreated or HRG treated versus neratinib-treated

The gene expression differences upon HRG treatment were sought to assess cellular response to this growth factor and investigate any differences between how HRG affects platinum-sensitive and platinum-resistant cell lines. HRG had different cellular functionality effects in PEA1 and PEA2 cells, hence the processes relating to the differential gene expression between the untreated controls and the HRG-activated cells in both cell lines were investigated. Neratinib inhibited proliferation, migration and invasion of the cell lines hence the pathways and biological processes affected by neratinib treatment were investigated in the absence or presence of HRG.

Differences between treatment groups were evaluated using the Rank Products method within TMeV, with FDR set at 5%, and these are depicted in Figure 6.3. In PEA1 cells, there were:

- 394 (2.4%) differently expressed genes in untreated control versus HRG-treated cells
- 439 (2.6%) differently expressed genes in untreated control versus neratinib-treated cells
- 434 (2.7%) differently expressed genes between the HRG-treated and HRG + neratinib-treated cells

Of these genes, there were 19 genes which were co-expressed in all three treatment groups. The overlapping genes were mostly relating to the MAPK pathway, featuring genes such as *RASGRP1*, *CACNG6*, *DUSP6*, *MAPK8IP1* and *TGFB3*. Other genes were related to TK signalling (genes including *HER3*, *IL7*, *GJC2*, *NGF*, *NTF3* and *SEMA3B*), and transcription (genes including many zinc finger proteins and *YBX2*). Neratinib induced proliferation-associated genes (through *WISP2*, *IGFBP1*, *SGK1* and *TMC8*) as well as genes associated with the MAPK pathway (through *C1QTNF2*, *CHRNA7*, *FGF1* and *LRRK2*). Neratinib treatment on HRG stimulated cells, also modulated expression of MAPK pathway (through *CXCR4*, *CHRFAM7A*, *DUSP5* and

FPR1), transcription (zinc finger proteins and *TRIB3*) as well as cell-cell signalling genes (*EFNB2, GJA3, IL11, NOV* and *TRHDE*).

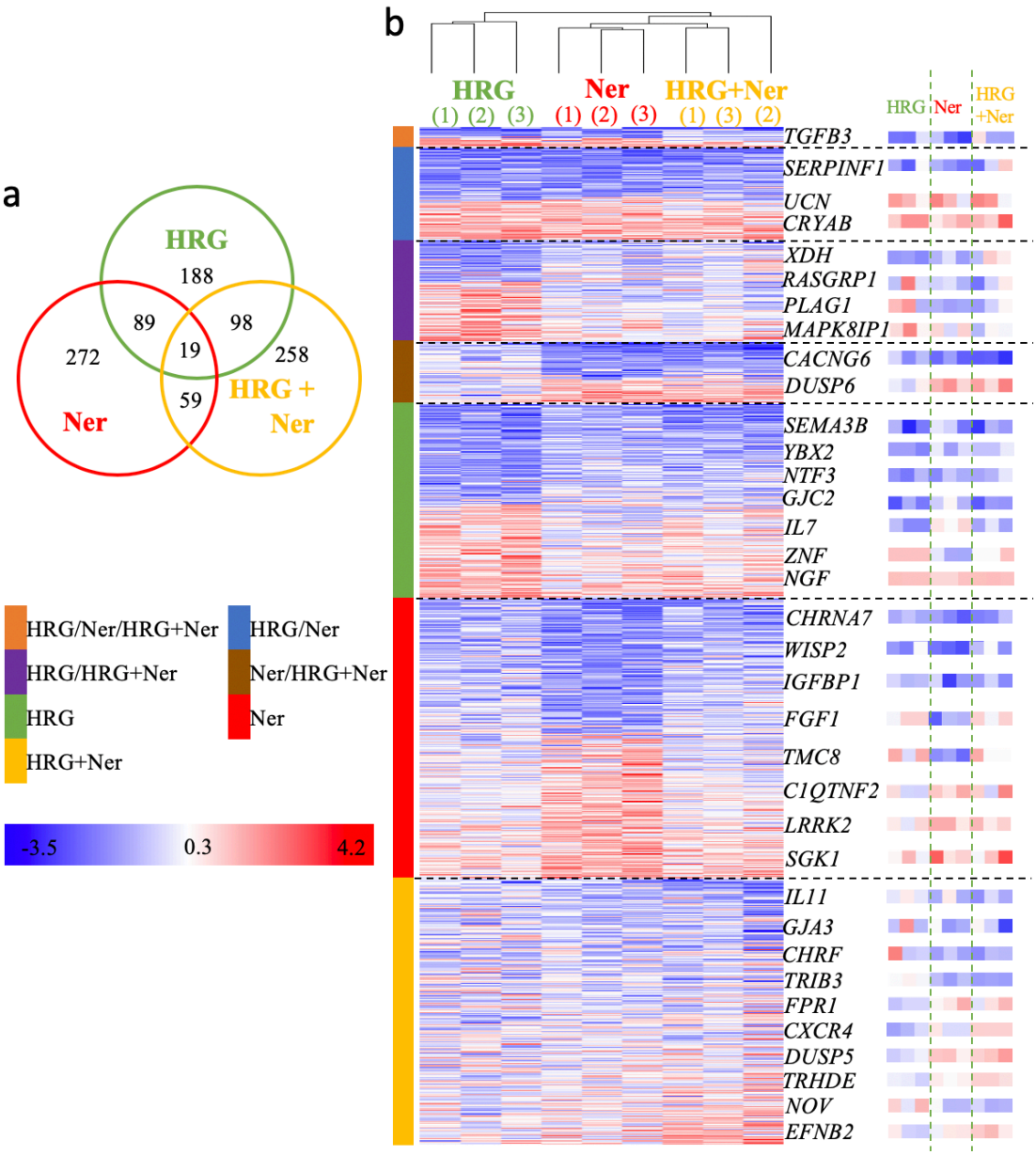


Figure 6.3: Differently expressed genes in PEA1 cells across different treatment groups

(a) number of differently expressed overlapping genes, (b) heatmap of the differently expressed genes in the different treatment groups and key genes associated within each group. The analysis was carried out using Rank Products with 5% FDR. The significant differently expressed genes within each group were defined in accordance with the DAVID online resource. HRG/Ner/HRG+Ner represents genes that were co-expressed between the 3 treatment groups, HRG/Ner represents genes that were co-expressed between HRG and neratinib treatment groups, HRG/HRG+Ner represents co-expressed genes in HRG treated and HRG+neratinib treated, Ner/HRG+Ner represents the overlapping genes in neratinib and HRG+neratinib treatments, while HRG represents genes that were only differently expressed upon HRG stimulation, Ner represents the genes differently expressed only upon neratinib

inhibition and HRG+Ner represents the genes that are differently expressed upon HRG and neratinib treatment.

Figure 6.4 depicts the differently expressed genes in PEA2 cells, and there were:

- 410 (2.5%) differently expressed genes in untreated control versus HRG-treated cells
- 458 (2.8%) differently expressed genes in untreated control versus neratinib-treated cells
- 434 (3.1%) differently expressed genes between the HRG-treated and HRG + neratinib-treated cells

In PEA2 cells, the co-expressed differently expressed genes were mostly related to the FoxO pathway (*TNFSF10, CCND1, SGK2, MAPK12, PCK2,* and *PIK3R3*), and MAPK as well as PI3K-Akt pathway (*ATF4, NR4A1, MYC, EPHA2, GHR, DUSP5, TNF,* and *DUSP6*). Stimulation with HRG modulated expression of transcription genes (such as *INHBA, LHX2* and zinc finger proteins). Neratinib inhibition affected genes of the PI3K-Akt pathway (*CDKN1A, EPOR, IRS1,* and *IL6*). On the other hand, neratinib treatment and HRG stimulation mainly affected transcription (*MMP9, HMGA2, DDIT3*), PI3K-Akt and MAPK pathway (*LAMC3, KITLG, JAK3, NGF, ITGA2B, DUSP2, HSPA2*) as well as the p53 pathway (*CCNE2, TP53, SESN2, CCNG2*).

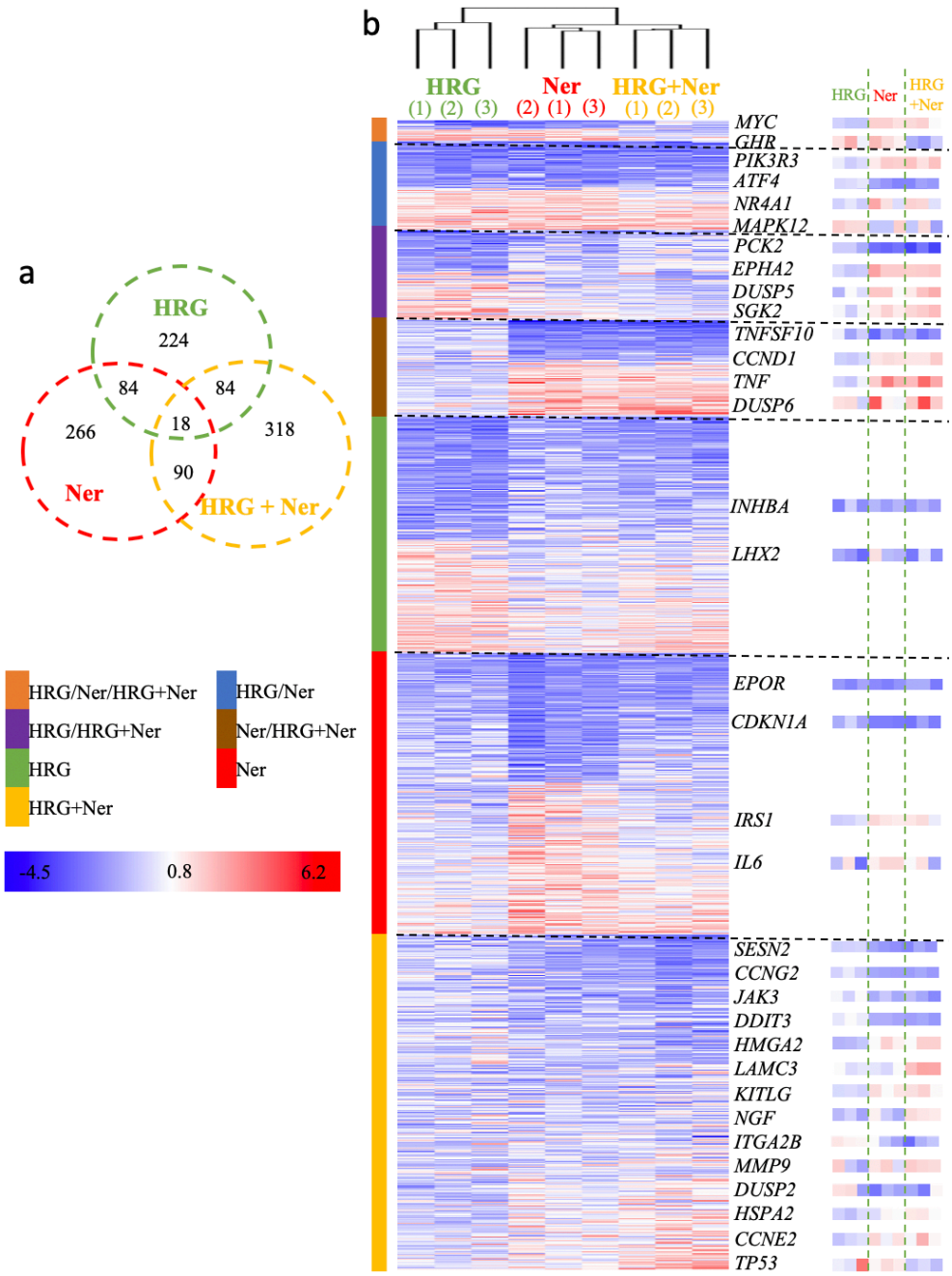


Figure 6.4: Differently expressed genes in PEA2 cells across different treatment groups

(a) number of differently expressed overlapping genes, (b) heatmap of the differently expressed genes in the different treatment groups and the key genes associated within each group. The analysis was carried out using Rank Products with 5% FDR. The significant differently expressed genes within each group were defined in accordance with the DAVID online resource. HRG/Ner/HRG+Ner represents genes that were co-expressed between the 3 treatment groups, HRG/Ner represents genes that were co-expressed between HRG and neratinib treatment groups, HRG/HRG+Ner represents co-expressed genes in HRG treated and HRG+neratinib treated, Ner/HRG+Ner represents overlapping genes in neratinib and HRG+neratinib treatments, while HRG represents genes that were only differently expressed upon HRG stimulation, Ner represents the genes differently expressed only upon neratinib inhibition and HRG+Ner represents the genes that are differently expressed upon HRG and neratinib treatment.

To support the analysis of genes that change upon HER-targeted therapy, the publicly available Monks *et al.* dataset [446], featuring HGSOC cell lines (OVCAR3, OVCAR4, OVCAR5 and OVCAR8), treated with lapatinib for 6 h, was chosen. The differently expressed genes upon treatment were evaluated using TMeV, at 1% FDR. At such stringent criteria, there were over 120 differently expressed genes that were co-expressed between at least one neratinib-treated cell line and one lapatinib-treated cell line (Figure 6.5). The most significant pathways that these overlapping genes represented included the TNF, FoxO and mTOR pathway.

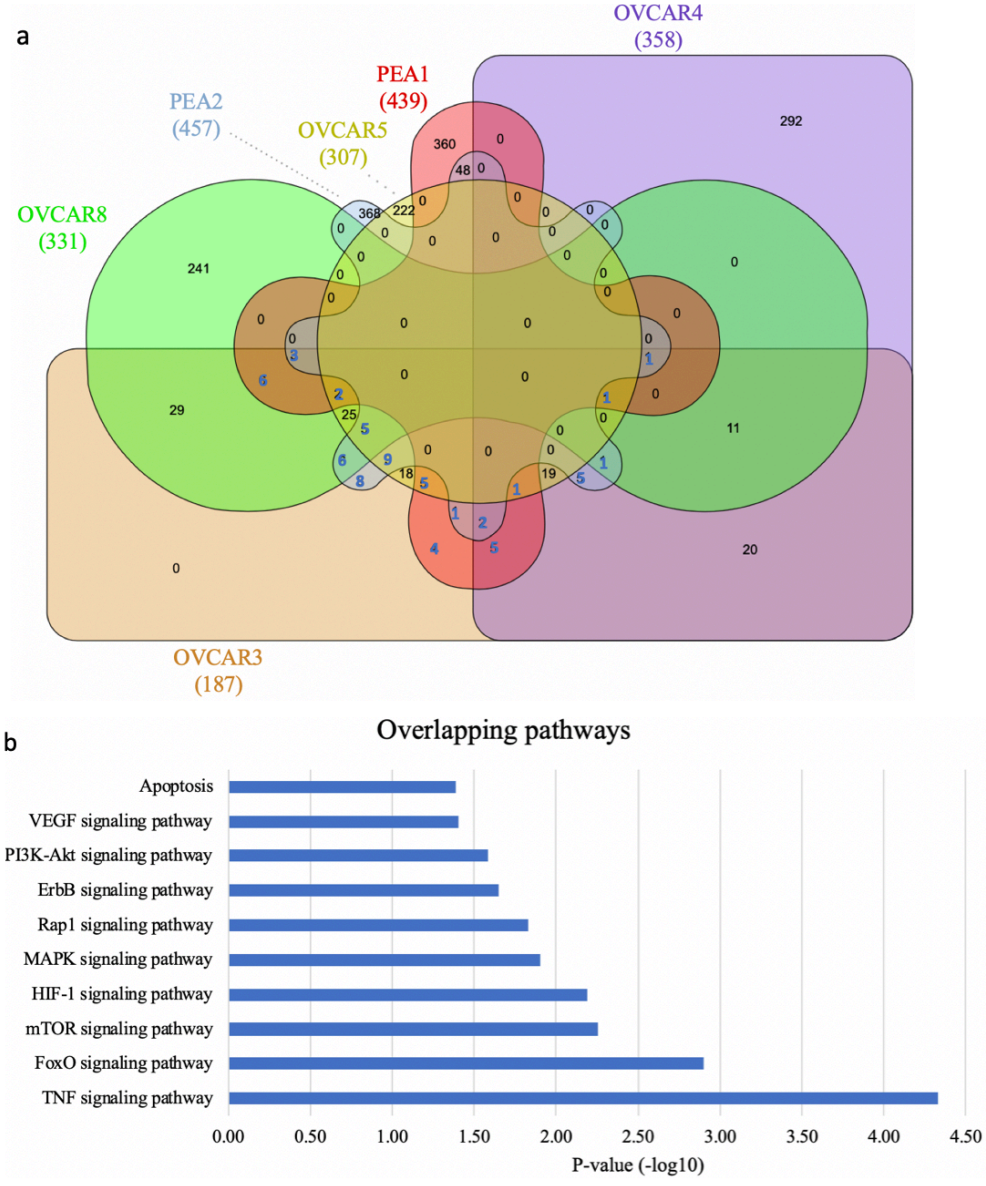


Figure 6.5: Differently expressed genes in lapatinib-treated HGSOC cell lines

(a) *Venn diagram of differently expressed genes between lapatinib-treated HGSOC cell lines and neratinib-treated HGSOC cell lines. (b) The pathways related to the overlapping*

differently expressed genes in lapatinib and neratinib treated HGSOC cell lines. The differently expressed genes in dataset GSE116436 [446] were evaluated using FDR of 1%. The untreated control data were compared against 1 μM lapatinib treatment for 6 h. The genes taken into consideration for pathway evaluation were the genes which had at least one lapatinib-treated and one neratinib-treated cell line involvement. The pathways were analysed using DAVID online resource and the most significant ones are shown.

6.3 HER-targeted therapy-induced biomarkers

To identify predictive biomarkers which might be used to assess response to HER-targeted treatment, previous gene expression data containing information about the effect of trastuzumab, pertuzumab or their combination on SKOV3 ovarian cancer xenografts was mined [232]. The gene expression data for combined pertuzumab and trastuzumab therapy after four days of treatment were compared against the untreated control to explore the differently expressed genes using the Rank Product method in TMeV. This treatment group was considered as it produced the best growth-inhibitory effects against the ovarian cancer xenografts tested [232, 233]. The differently expressed genes were compared with the differently expressed genes that resulted upon neratinib treatment in the PEA1 and PEA2 cell line pair discussed in section 6.2, and the co-expressed genes were taken into consideration (Figure 6.6).

There were 13 genes that were down-regulated and co-expressed between mAb-treated xenografts and at least two neratinib-treated groups (Figure 6.6a). On the other hand, there were 24 genes that were up-regulated and co-expressed in at least two neratinib-treated groups and mAb-treated xenografts (Figure 6.6b). Literature searching was used to help determine which of these genes were of greatest interest that could be explored at the protein level using TMAs. The criteria for the gene choice included the novelty of the genes in the ovarian cancer setting yet with enough being known about the genes, as well as the availability of antibodies to the proteins for the genes of interest. The TMAs constructed consisted of SKOV3 ovarian cancer tumour cores that were treated with trastuzumab, pertuzumab or the combination sampled over a period of days from the study by Sims *et al*. [232]. The proteins that were investigated further were: *CEBPG* (CCAAT Enhancer Binding Protein Gamma), *DDIT4L* (DNA Damage Inducible Transcript 4 Like), *RALGDS* (Ral guanine nucleotide dissociation stimulator), and *SPRY2* (Sprouty homolog 2).

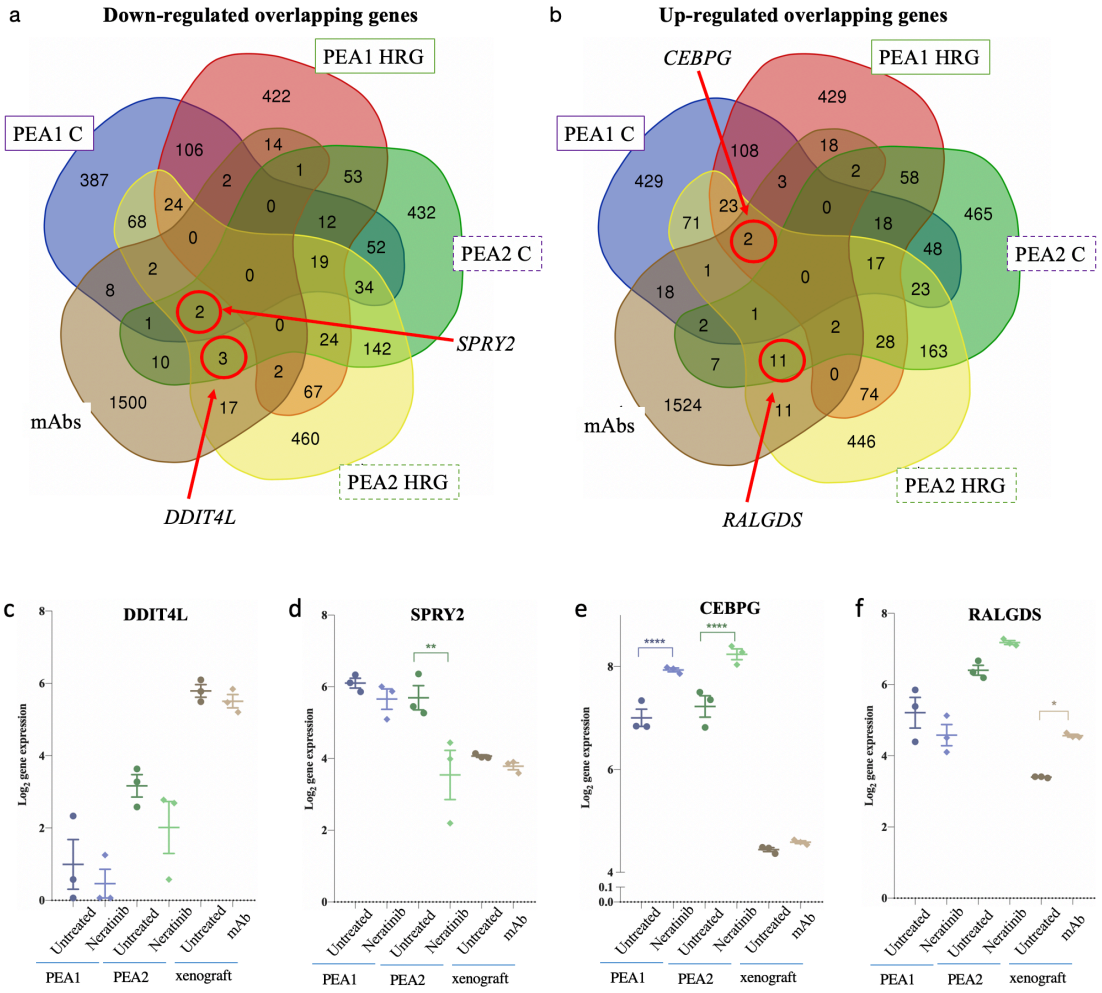


Figure 6.6: Venn diagram with co-expressed genes between neratinib-treated cell lines and mAb-treated xenografts

(a) Down-regulated overlapping genes between PEA1 cell line neratinib control, PEA1 cell line HRG-neratinib treated, PEA2 cell line neratinib control, PEA2 cell line HRG-neratinib treated, and ovarian cancer xenografts treated with combination of pertuzumab + trastuzumab. (b) Up-regulated overlapping genes between PEA1 cell line neratinib control, PEA1 cell line HRG-neratinib treated, PEA2 cell line neratinib control, PEA2 cell line HRG-neratinib treated, and ovarian cancer xenografts treated with combination of pertuzumab + trastuzumab, (c-f) gene expression of the 4 HER-therapy induced candidates in PEA1 and PEA2 untreated and neratinib-treated and in the xenograft model in the controls and combination-treated, where (c) DDIT4L, (d) SPRY2, (e) CEBPG, and (f) RALGDS. The gene expression data for mAb xenografts were taken from Sims et al. [232].

6.3.1 Expression of the therapy-induced biomarkers in platinum-sensitive vs platinum-resistant tumours

The TCGA gene expression dataset for HGSOC [447] was analysed to investigate differences in the therapy-induced biomarker expression in platinum-sensitive versus platinum-resistant ovarian cancer (Figure 6.7). Platinum status of the patients in this

dataset were based on the disease-free status versus progression within six months after completing platinum-based therapy for the platinum-sensitive and platinum-resistant classification respectively [447]. The gene expression levels of *CEBPG*, *RALGDS*, *SPRY2* and *DDIT4L* were exported from the dataset across platinum-sensitive and platinum-resistant tumour expression. *CEBPG* and *DDIT4L* were more highly expressed in the platinum-resistant samples. On the other hand, *RALGDS* and *SPRY2* had higher expressions in the platinum-sensitive samples.

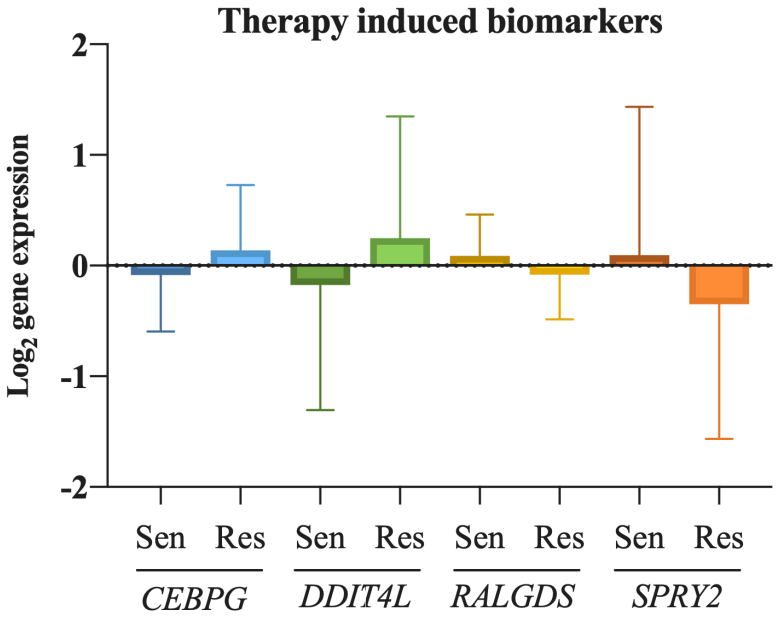


Figure 6.7: Therapy-induced biomarkers in platinum-sensitive and platinum-resistant tumours

The TCGA data [447], were accessed and CEBPG, DDIT4L, RALGDS and SPRY2 expression information of platinum-sensitive and platinum-resistant tumours was extracted. The differences in expression were not statistically significant. "Sen" means sensitive and "Res" means resistant.

6.3.2 Patient survival analysis of therapy-induced biomarkers

The impact of *CEBPG*, *DDIT4L*, *RALGDS* and *SPRY2* gene expression on PFS and OS of serous ovarian cancer patients was investigated using KMplot[6] [448]. Kaplan-Meier plots show that high expression of *CEBPG* and *DDIT4L* are linked with significantly worse PFS and OS, while low expression of *RALGDS* and *SPRY2* are associated with worse PFS and OS (Figure 6.8).

[6] KMplot online resource. Available from: https://kmplot.com/analysis/

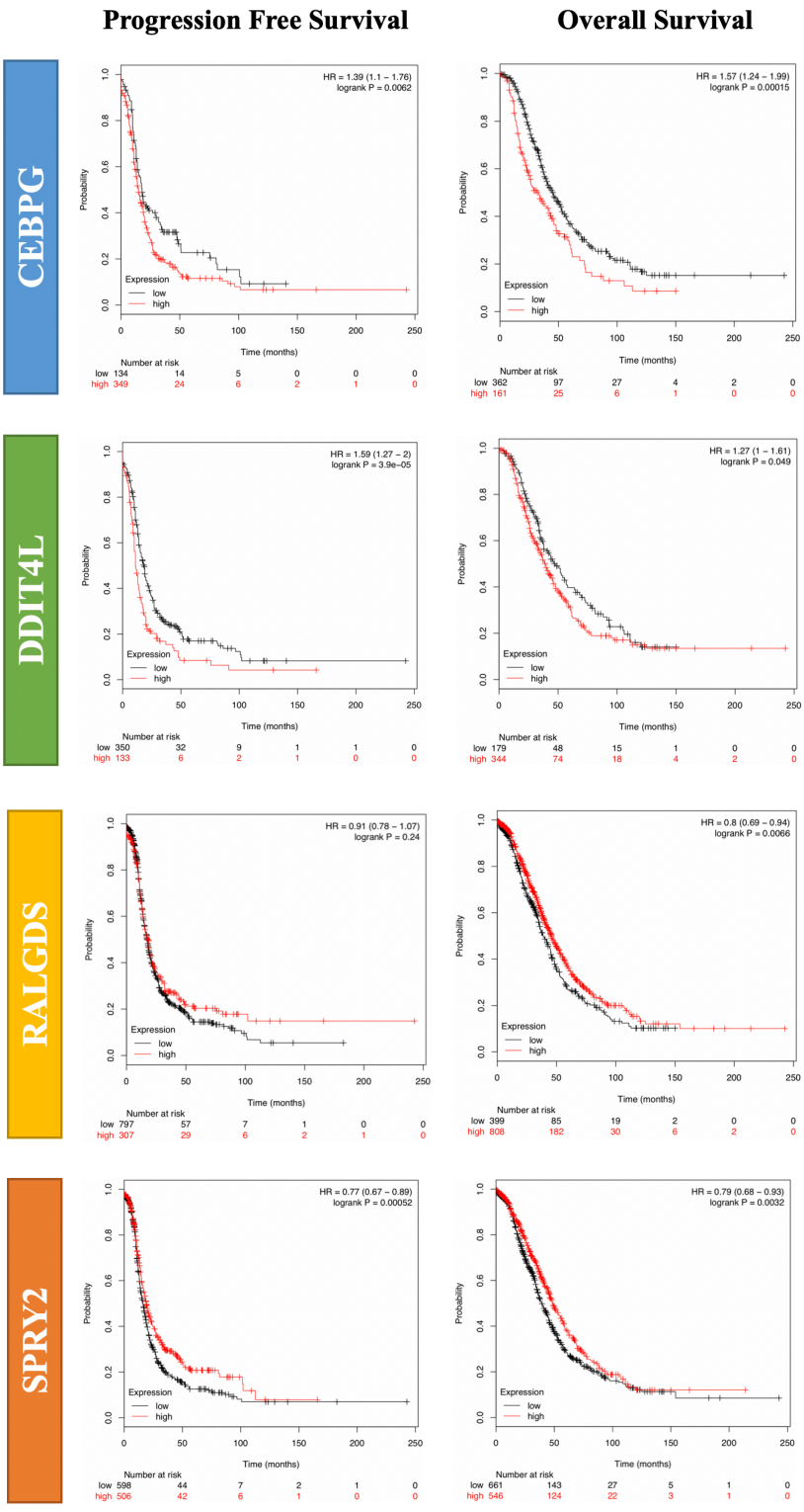
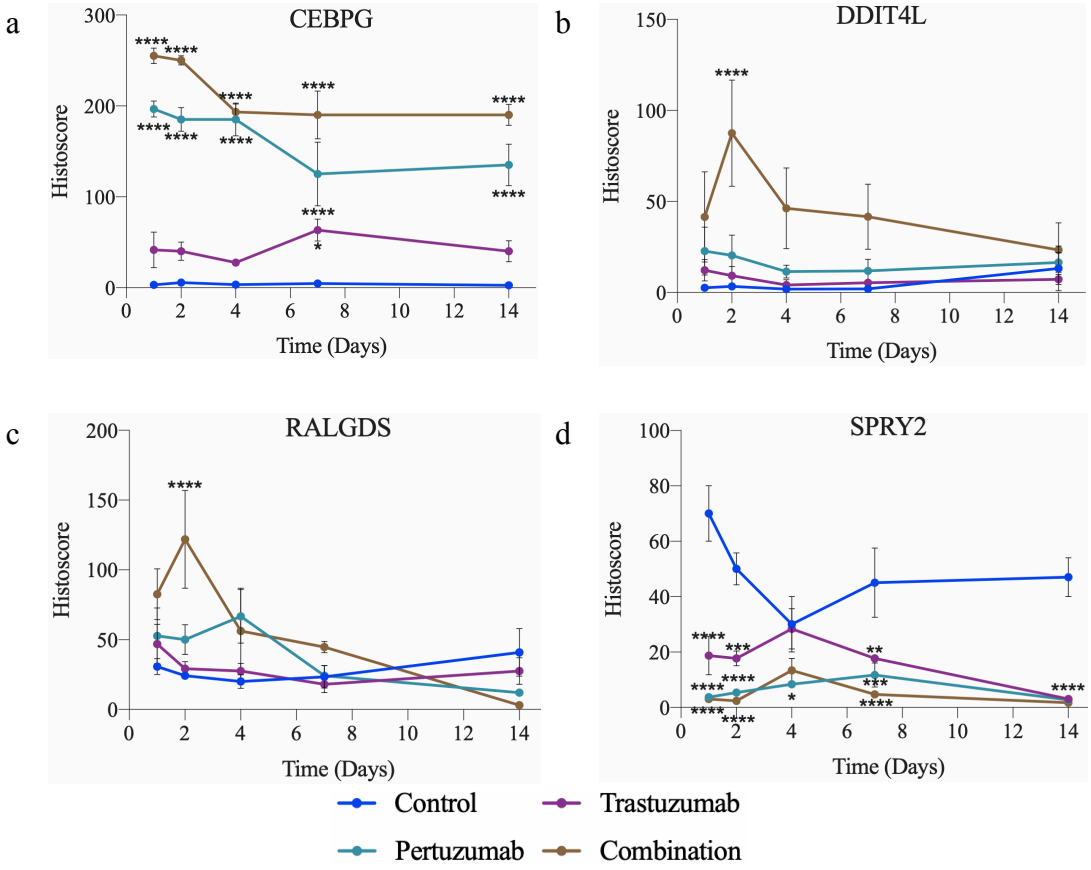


Figure 6.8: Kaplan-Meier plots for progression free survival and overall survival for CEBPG, DDIT4L, RALGDS and SPRY2

The online resource KMplot [448] was used to perform survival analysis for CEBPG, DDIT4L, RALGDS and SPRY2 expression in serous ovarian cancer samples across all cancer grades over 240 months. Samples were placed into high (red line) and low (black line) expression groups based on the optimum cut-point in each individual analysis. Each plot shows the p-value, hazard ratio and the number of patients in the analysis.

6.3.3 Treatment-induced expression of biomarkers

The selected treatment-induced biomarkers identified from the co-expression between differently expressed genes upon neratinib treatment and differently expressed genes upon mAb treatment were assessed in TMAs containing xenograft tumours treated with pertuzumab, trastuzumab or the combination, over a 14-day period. Each treatment group consisted of three tumour replicates and there were four technical replicates. The expression levels of CEBPG, DDIT4L and RALGDS proteins were highest in tumours containing combination treatment of mAbs, followed by pertuzumab-treated samples and trastuzumab-treated samples. The untreated controls had the lowest protein expression. On the contrary, SPRY2 protein expression was lowest in the mAb combination-treated and pertuzumab-treated samples, and highest in the untreated control. The protein expression usually peaked around day 2-4 of treatment and gradually decreased after that.



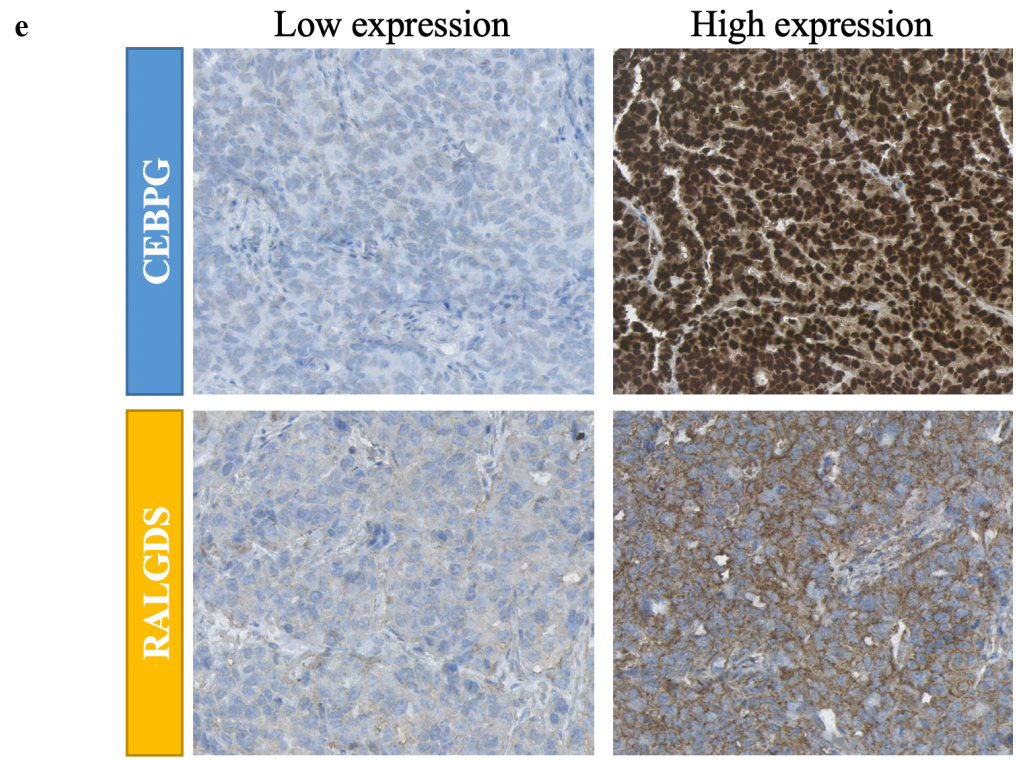


Figure 6.9: Expression of biomarkers upon mAb treatment in TMAs containing xenograft tumour samples

*(a) expression of CEBPG protein, (b) expression of DDIT4L protein, (c) expression of RALGDS protein, (d) expression of SPRY2 protein, and (e) low and high staining with CEBPG and RALGDS antibodies. Histoscores were measured by multiplying the % of stained cells by the intensity of staining. Results represent average from 3 technical replicates from 4 TMAs and error bars signify SEM. Statistical analysis was performed against the untreated controls using two-way ANOVA (where *P≤0.05, **P≤0.01, and ****P<0.0001).*

6.4 Discussion

In previous chapters, the results obtained showed differences between the platinum-sensitive and platinum-resistant cell lines in their response to therapy. The purpose of this chapter was to investigate gene and protein expression differences using a data-driven molecular approach between phenotypes and upon treatment and how these link to *in vitro* data.

Gene expression analysis revealed that in the platinum-resistant cell line PEA2, expression of the ECM-receptor interaction, focal adhesion and PI3K-Akt signalling pathways were down-regulated pathways represented by the differently expressed genes over PEA1 cell line. These pathways are associated with the cell cycle and cell migration biological processes. Genes identified that were decreased and related to cell cycle included *CDKN2A* (cyclin-dependent kinase Inhibitor 2A), which acts as a tumour suppressor [449], *FOXL2* (Forkhead Box L2) which is involved in ovarian development [450] and *MYC* (MYC proto-oncogene), a proto-oncogene which is associated with cell progression and transformation [451]. On the other hand, genes related to cell migration included *MET* (MET proto-oncogene), which plays a role in cell survival and invasion [452], and *ENG* (endoglin), which has a critical role in angiogenesis [453]. In breast cancer, increased *MET* expression has been associated with anthracycline-based chemotherapy resistance [452]. The expression of these biological processes ties in with the *in vitro* results, whereby the PEA1 cell line had faster migration rate than the PEA2 cell line.

On the other hand, the differentially expressed genes in the platinum-resistant cell line were linked with the Wnt signalling and the MAPK pathways. The Wnt signalling pathway is involved in cellular events, such as proliferation, survival, migration and polarity. Previous studies have also suggested that Wnt activity plays an important role in the emergence of chemo-resistant ovarian cancer, with the aid of endothelin-1, endothelin A receptor which regulates EMT [454, 455]. Wnt5a has also been identified in previous studies to be associated with decreased chemosensitivity [456], which fits well with the findings in this project, as PEA2 cells had higher Wnt5a expression, and are less chemo-sensitive than their counterpart PEA1 cells. Elevated CTNNB1 levels

in ovarian cancer correlate with improved patient outcomes, and Wnt5a activity generally has negative effects on CTNNB1 [457]. Apart from Wnt signalling, cell adhesion is also a biological process which was differently expressed in the PEA2 compared to the PEA1 cell line. In fact, the PEA2 cell line adhered more than PEA1 cell line to the ECM proteins as seen in Chapter 3. Additionally, *HER2*, *HER3* and *HER4* genes were more highly expressed in the PEA2 cell line, a result which was consistent with the protein expression levels using western blot analysis. Interestingly, a previous study by Koti *et al.* identified how amongst others, the MAPK pathway was over-represented in the platinum-resistant HGSOC, in addition to increased IGF1 and NF-κB expression [458].

Stimulation with HRG caused an up-regulation in genes linked to transcription mis-regulation in both PEA1 and PEA2 cell lines. Mutations in the regulatory regions and transcription factors, are main contributors of cancer. In fact, the transcription factor *MYC*, which was differently expressed in most instances in PEA1 and PEA2 cell lines, is frequently amplified, and is associated with tumour aggression and poor clinical outcome [459]. Previous studies suggest that HRG-mediated MAPK can stimulate transcription of the cyclin D1 gene [460], which is required for the cell cycle progression through G1 phase [461]. It is important to note that gene expression levels do not always correlate well with protein levels as multiple factors influence levels of expression of both mRNA and proteins, including regulation of transcription to translation, mRNA half-life as well as post-transcriptional modification of proteins [462].

Addition of neratinib modulated expression of FoxO signalling pathway genes. This family of transcription factors regulate expression of genes associated with cellular physiology, including apoptosis, glucose metabolism and oxidative stress resistance [463]. In gastric cancer, *FoxO1* is an important link between HER2 and MET signalling pathways through negative crosstalk, and was found to be an important component in acquired lapatinib resistance in HER2 positive gastric cancer cells [464]. This finding links well with this data considering that *MET* was upregulated in the TKI-resistant cell line PEA1, and differential gene expression identified that FoxO signalling is down-regulated in PEA1 cells.

Generally, neratinib caused down-regulation of components of the MAPK and PI3K pathways, which are downstream of the HER receptors, even in the presence of HRG. The inhibition of elements of the MAPK and PI3K pathway was in line with the protein expression analysis carried out by western blot in Chapter 4, where neratinib was able to decrease the phosphorylation of ERK and Akt, which are products of the MAPK and PI3K pathways respectively. Some of the most influential genes in the MAPK pathway inhibition would be the DUSP family, as they are usually negative feedback regulators of ERK [465].

Prior studies by O'Neill *et al.* have shown that expression of the *CCND1* gene is usually down-regulated in response to lapatinib, neratinib and afatinib treatment [466]. This was also observed in this dataset, whereby *CCND1* was down-regulated in both cell lines, even in HRG-modulated cells. The study by O'Neil *et al.* also identified increased expression of *RB1CC1* (RB1 Inducible Coiled-Coil 1), *HER3*, *FoxO3a* and *NR3C1* (Nuclear Receptor Subfamily 3 Group C Member 1) upon treatment with lapatinib, neratinib and afatinib in breast cancer cell lines [466]. These genes are usually indicative of a HER2 pathway connection, which was consistent with gene expression changes using gefitinib (EGFR-specific TKI), which did not cause differential gene expression in these genes [466]. In this data set, *HER3* expression was increased in only the PEA1 neratinib control, while *RB1CC1*, *FoxO3a* and *NR3C1* were not found to be differentially expressed upon neratinib treatment.

6.4.1 Potential biomarkers of anti-HER family treatment

Biomarkers can include genes or proteins which are used to identify pathological or physiological processes or diseases [467]. A biomarker may be prognostic or predictive. Prognostic biomarkers deliver information about the patient's outcome to cancer, whilst predictive biomarkers provide information about the patient's response to therapeutic interventions, and can also be a target for targeted therapy [468].

Previous studies by Sims *et al.* using the SKOV3 ovarian cancer xenograft model revealed that the combination of mAbs was associated with increased apoptosis, as seen through the enhanced expression of cleaved caspase 3 [232]. The same study also

observed that pertuzumab was capable of reducing p-Akt, while both monoclonal antibodies singly and their combination reduced p-ERK expression after 4 days [232].

The protein expression changes in the TMAs complemented the gene expression changes observed upon mAb therapy, whereby the CEBPG and RALGDS genes or proteins increased upon treatment, whilst SPRY2 genes or proteins decreased upon treatment. However, DDIT4L protein expression increased upon treatment in the TMAs, while the gene expression level (at Day 4) decreased upon treatment. These discrepancies could be due to gene transcription changes at Day 4 not reflecting the overall dynamic change in protein expression. The expression of the biomarkers is associated with better survival in the platinum-sensitive disease. CEBPG and DDIT4L were less expressed in the platinum-sensitive cells than the platinum-resistant cells, which was associated with better PFS and OS. Conversely, RALGDS and SPRY2 were more highly expressed in the platinum-sensitive cells, which was similarly associated with better PFS and OS over low expression of biomarkers.

The *CEBPG* protein mediates cellular transcription involved in the metabolism and differentiation of cells [469]. This protein is also thought to positively regulate wound repair through its effects on the HER pathway [470]. In NSCLC, CEBPG expression has been associated with radio-sensitivity [471], while in ovarian cancer, it is modulated in response to paclitaxel treatment [472]. There are other members of the CEBP family, including CEBPB, which has two isoforms: CEBPB LIP and CEBPB LAP, whose ratio correlates to chemotherapy response. It was found that degradation of LIP causes endoplasmic reticulum stress and chemotherapy-induced cell death [473]. Another member, CEBPD is associated with the amplification of IL-6 and HIF-1 signalling, which in turn link to inflammation and hypoxia [474].

DDIT4L (also referred to as REDD2) is a paralog of DDIT4 [475], and is upregulated under cellular stress, such as hypoxia, endoplasmic reticulum stress and starvation [476-478]. *DDIT4L* and *DDIT4* are hypoxia-inducible factor (HIF)-1 inducible genes and their presence inhibits mTOR [478-480]. In ovarian cancer studies, DDIT4L gene upregulation caused a decrease in size of cells [481]. In this study, the Kaplan-Meier plots show that high expressions of DDIT4L are associated with poor OS and PFS.

The Ras pathway which was up-regulated by neratinib treatment, has downstream effectors including PI3K, Raf and Ral-GEF [482]. The latter includes proteins that are regulated by the *RALGDS* gene. RALGDS is normally involved with golgi trafficking vesicle formation [482]. RALGDS and Akt contribute to the inhibition of FoxO family [483]. *In vitro* studies investigating the effects of lapatinib in HER2+ cancer cells over a short period time, showed that after 12 h, phospho Raf was activated with lapatinib treatment [484]. It is feasible therefore that there might be a lapatinib-mediated FoxO-dependent feedback loop, activating the Raf pathway [484]. High levels of RALGDS were associated with better PFS and OS in the Kaplan-Meier plots.

The sprouty family consist of four homologs, thought to be negative pathway regulators of the Ras/ERK pathway [485, 486]. SPRY2 can inhibit MAPK pathway signalling by sequestering CBL (Cas-BR-M murine ectopic retroviral transforming sequence homolog), which causes EGFR degradation [485, 487]. Expression of SPRY1 correlated with SPRY2 expression and were associated with survival in ovarian cancer patients [488]. Additionally, SPRY2 expression is correlated with tumour suppressor properties in cancer, such as proliferative, migratory, invasive and survival assets [486, 489-491]. In breast cancer, low expression of SPRY2 was linked with poor outcomes in trastuzumab-treated patients [492], which suggest that SPRY2 might be a useful biomarker to stratify patients for mAb therapy. Gene expression studies upon neratinib and mAb treatment showed that SPRY2 was down-regulated, which suggests that the activity of neratinib and mAbs might involve the tumour suppressor effect of SPRY2.

In summary, this chapter sought to investigate the pathways and processes that neratinib influences upon treatment using a molecular data-driven approach. The role of neratinib was also compared in the absence and presence of HRG. The findings corroborate the results in Chapter 3 and 4, whereby neratinib inhibited proliferation, migration and invasion even in the presence of HRG. Furthermore, the MAPK and PI3K pathways were also inhibited which was shown in Chapter 4 through inhibition of p-ERK and p-Akt in western blots. Additionally, this chapter has also brought together gene expression changes upon neratinib treatment with HER-targeted mAb treatment. Several therapy-induced biomarkers were evaluated and the combination of

pertuzumab and trastuzumab produced greatest changes in expression, followed by pertuzumab. This profile was observed in the proliferation and migration studies from Chapter 3, whereby collectively, the combination of antibodies and pertuzumab had greater inhibitory effects than trastuzumab alone.

7 Conclusion

The central aim of this project was to assess the therapeutic effects of HER-targeted inhibitors in platinum-sensitive compared to platinum-resistant HGSOC cell lines, and to evaluate the role of HER family receptor expression (and presence of activating ligand) in the efficacy of these drugs. In this project, the effects of TKIs were compared with the inhibitory effects of HER-targeting mAbs, as monotherapy as well as their potential in combination approaches. Additionally, this undertaking sought to identify potential HER-targeted therapy-induced biomarkers, whose expression changed upon neratinib-treatment and assessment of these proteins in tissue microarrays consisting of ovarian cancer xenografts treated with mAbs was carried out. To our knowledge, this is the first study to assess the role of pan HER-targeted TKIs in clinically derived platinum-sensitive and platinum-resistant pairs of HGOSC cell lines. One of the pairs of cell lines represented a relapsed platinum-sensitive ovarian cancer case, while the other two pairs represented platinum-naïve ovarian cancer instances. Inhibitor effects on proliferation, migration, invasion, adhesion and cell signalling using both 2D and 3D cancer cell models in the presence or absence of EGF and HRG ligands were investigated.

Initial results comparing the platinum-sensitive and platinum-resistant cell lines showed that the platinum-sensitive counterparts, particularly in the case of PEA1-PEA2 cell line pair, had increased EGFR and HER3 expression, and greater proliferation and migration abilities than their platinum-resistant counterparts. On the other hand, the platinum-resistant counterpart had greater HER2 and HER4 expression, enhanced adherence potential and was more invasive, which was generally similar in the other two cell line pairs. These findings were consistent with the gene expression analysis conducted on the PEA1 and PEA2 cell line pair, whereby the

PEA1 cell line had higher gene expression levels of components of the cell cycle and migration processes, whilst the PEA2 cell line had increased gene expression levels of molecules linked to adhesion and HER2 and HER4 expressions.

7.1.1 TKIs in combination with cytotoxic or mAb agents augment inhibitory responses

Previous studies have suggested that platinum-resistant cells have increased sensitivity to pertuzumab in ovarian cancer patients, especially in the presence of low HER3 levels [230, 231]. In the current study, the TKIs were effective in their anti-proliferative, anti-migratory, anti-invasive and anti-adhesive effects in all HGSOC cell lines. The most potent TKI was neratinib followed by sapitinib and afatinib. These three TKIs potently target three of the HER family receptors. Neratinib and afatinib target EGFR, HER2 and HER4, whilst sapitinib has greatest affinity for EGFR, HER2 and HER3 [147]. The mAbs produced limited inhibitory effects in comparison to the TKIs investigated, but similarly were more effective in the platinum-resistant counterpart. Pertuzumab produced the best anti-proliferative effects, whilst trastuzumab plus pertuzumab had the best anti-migratory effects.

Combination experiments assessing neratinib versus lapatinib in combination with cytotoxic agents and mAbs revealed that although generally neratinib had better additive and synergistic effects, combining lapatinib with another agent sensitised the cells to lapatinib treatment. Pre-treating cell lines with neratinib and adding cisplatin showed good synergistic effects, while the simultaneous addition of neratinib and pertuzumab had favourable results. Paclitaxel sensitised lapatinib's effect in the platinum-sensitive cell line PEA1 and produced synergistic effects in both scheduling treatments. Generally, simultaneous addition of TKIs and other agents produced better combinatorial effects.

7.1.2 TKIs inhibit stimulation by EGF and HRG

Additional experiments were undertaken to assess the activity of the TKIs in the presence of the natural ligands EGF and HRG, whereby the former binds to EGFR forming EGFR-based dimers whilst HRG binds to HER3 or HER4 to induce

dimerisation. Using western blot analysis, EGF caused more phosphorylation than HRG or the combination of EGF and HRG in the short-term (within 60 min). In longer-term incubations (over 48 h), HRG caused more stimulation of the MAPK pathway, than the PI3K pathway. The results showed that the TKIs were able to inhibit the activity induced by these ligands on the MAPK and PI3K pathways, at concentrations which are safely achievable in patients. Additionally, neratinib decreased the expression of HER2 and HER4 receptors, especially in the PEA2 and PEO4 cell lines.

EGF stimulated the migration and invasion rates of cell lines relative to the control (Figure 7.1), which was also observed in previous *in vitro* ovarian cancer studies, which showed that hyperactivity of EGFR is associated with enhanced motility, migration and invasion [493]. On the other hand, HRG decreased the invasion and migration capabilities of the cell lines. The presence of EGF decreased the sensitivity of the cells to neratinib and lapatinib in the anti-proliferative experiments. This decrease in anti-proliferative action of neratinib and lapatinib in the presence of EGF suggests that EGF activates EGFR-based dimerisations, and the TKIs are not fully capable of inhibiting the increased downstream effects. Alternatively, it may be that the TKIs do not rely on the EGFR solely for their anti-proliferative effects. On the other hand, the TKIs were still capable of inhibiting migration in the presence of EGF, to the same extent as to when ligands were not present. In certain cases, the presence of HRG decreased migration and invasion, especially in the PEA2 and PEO4 cell lines. These cell lines had a higher HER2 versus HER3/HER4 ratio, and this has been associated with inhibitory effects by the HRG ligand in previous studies [423]. Additionally, these two cell lines had increased expression of HER4 receptor in comparison to the other cell lines in this study. Hence, this could also suggest that the activation of HER3 and HER4 have inhibitory effects on migration and invasion.

A previous *in silico* model study to predict canertinib's sensitivity in epithelial ovarian tumours revealed that the best predictable model contained EGFR, HRG and at least another ligand, whereas HER2 decreased the accuracy of the model [285]. In the present study, the presence of HRG was generally associated with the cell lines being more sensitive to the anti-proliferative effects of TKI therapy, while EGF and EGFR did not affect sensitivity in a positive way. On the other hand, neratinib was able to

inhibit increased p-ERK and p-Akt expression that was brought about by the combination of EGF and HRG.

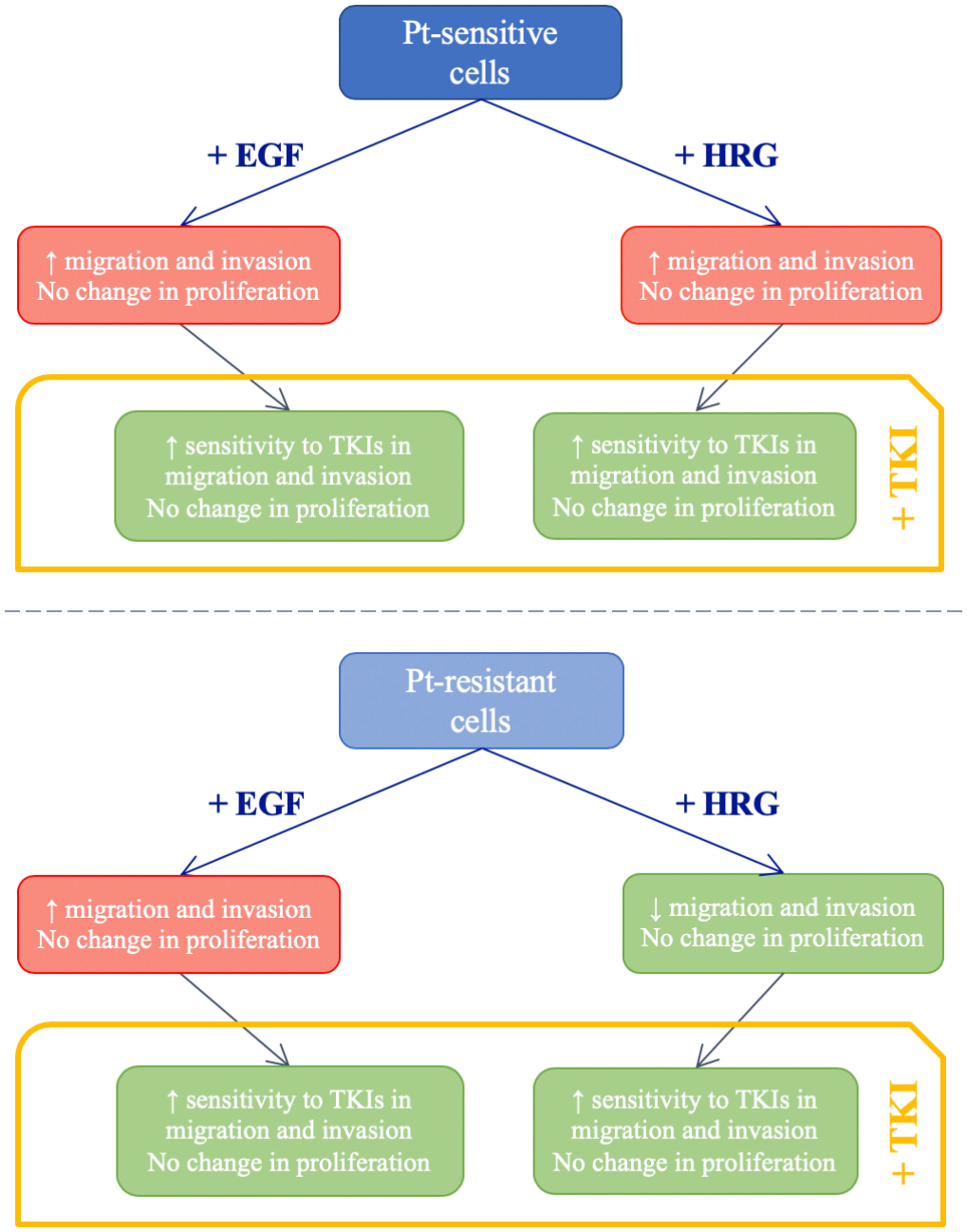


Figure 7.1: Summary of the effects of EGF or HRG on the platinum-sensitive and platinum-resistant HGSOC cell lines

This flow chart summarises relevant results upon the addition of the ligands - EGF or HRG - and presence of TKIs. The green boxes represent the favourable scenarios, whilst the red boxes represent the unfavourable situations in the cellular effects of proliferation, migration and invasion.

7.1.3 EGFR, HER2 and HER3 expression levels are important for TKI response

This project has also shown that the expression levels of the HER family members plays a crucial role in the sensitivity of cells towards the TKIs. HER2 and HER3 overexpression in these cell lines showed shifts in sensitivity to HER-targeted therapy in the sensitive versus the resistant cell lines. Increased HER2 and HER3 expression in the platinum-sensitive cell lines resulted in increased sensitivity to the HER-targeted TKIs than the WT cell lines. Conversely, higher HER2 and HER3 expression in the platinum-resistant cell lines caused decreased sensitivity to the HER-targeted TKIs than the WT cell lines. These results could suggest that increasing HER2 and HER3 expression in the platinum-sensitive cell lines creates the maximum level of receptors needed for the full action by the TKIs. Contrarily, too high a level of HER2 and HER3 expression in the platinum-resistant cell lines result in the TKIs being unable to bind to all receptors due to receptor over-saturation. There was no direct correlation between the HER2 and HER3 expression in these cell lines, and the sensitivity of TKIs. In platinum-resistant/refractory ovarian cancer patients, there was no association between baseline HER2 and HER3 expression and canertinib potency in the patients studied [284]. Previous studies suggest that overexpression of HER3, causes sustained PI3K/Akt signalling, which is an important mechanism of HER-targeted therapy resistance [494, 495]. Additionally, HER3 overexpression has been associated with acquired trastuzumab resistance in ovarian epithelial adenocarcinomas [496].

The expression level of HER2 and HER3 might not be a straightforward way of predicting sensitivity to therapy in HGSOC cell lines, however, it might offer some predictability if platinum-sensitive and platinum-resistant cases were considered as distinct stages of the disease. Additionally, it supports the possibility that the ratios of HER receptors are crucial for the optimum therapeutic potential of HER-targeted therapy [423].

For EGFR expression, neratinib inhibition of growth was not affected by the decreased expression of EGFR. In fact, the anti-proliferative effects of neratinib remained very similar as to when EGFR expression was present in the cells. On the other hand, low

EGFR levels reduced the efficacy of neratinib in its anti-migratory effects. This suggests that for neratinib's mode of action, the presence of EGFR is more important for the anti-migratory rather than anti-proliferative effects. Similarly to the study by Nagumo *et al*., the presence of EGF did not produce a more favourable environment for the anti-proliferative effects of pertuzumab in inhibiting the proliferation of ovarian cancer cell lines [417]. However, HRG presence increased the sensitivity of the cell lines towards pertuzumab. The enhanced sensitising effect HRG produces in the presence of pertuzumab was also noticed in this study, whereby the anti-proliferative effects were generally greater in the presence of this ligand, and all the mAbs were able to inhibit the phosphorylation produced by HRG stimulation but not EGF stimulation. This further supports the hypothesis that the HER3 to HER2 ratio is important for the sensitivity of HER-targeted therapy.

Platinum-resistance in ovarian cancer has been attributed to activated MAPK and PI3K pathways [497]. In fact, in previous studies whereby HER-targeted or MAPK inhibitors were used in combination with platinum agents, these combinations showed increased sensitivity towards platinum agents [357, 498, 499]. In this study, it was also evident that the HER pathway was frequently more activated in the platinum-resistant counterpart, providing at least one mechanism as to why these TKIs were more effective in the platinum-resistant HGSOC.

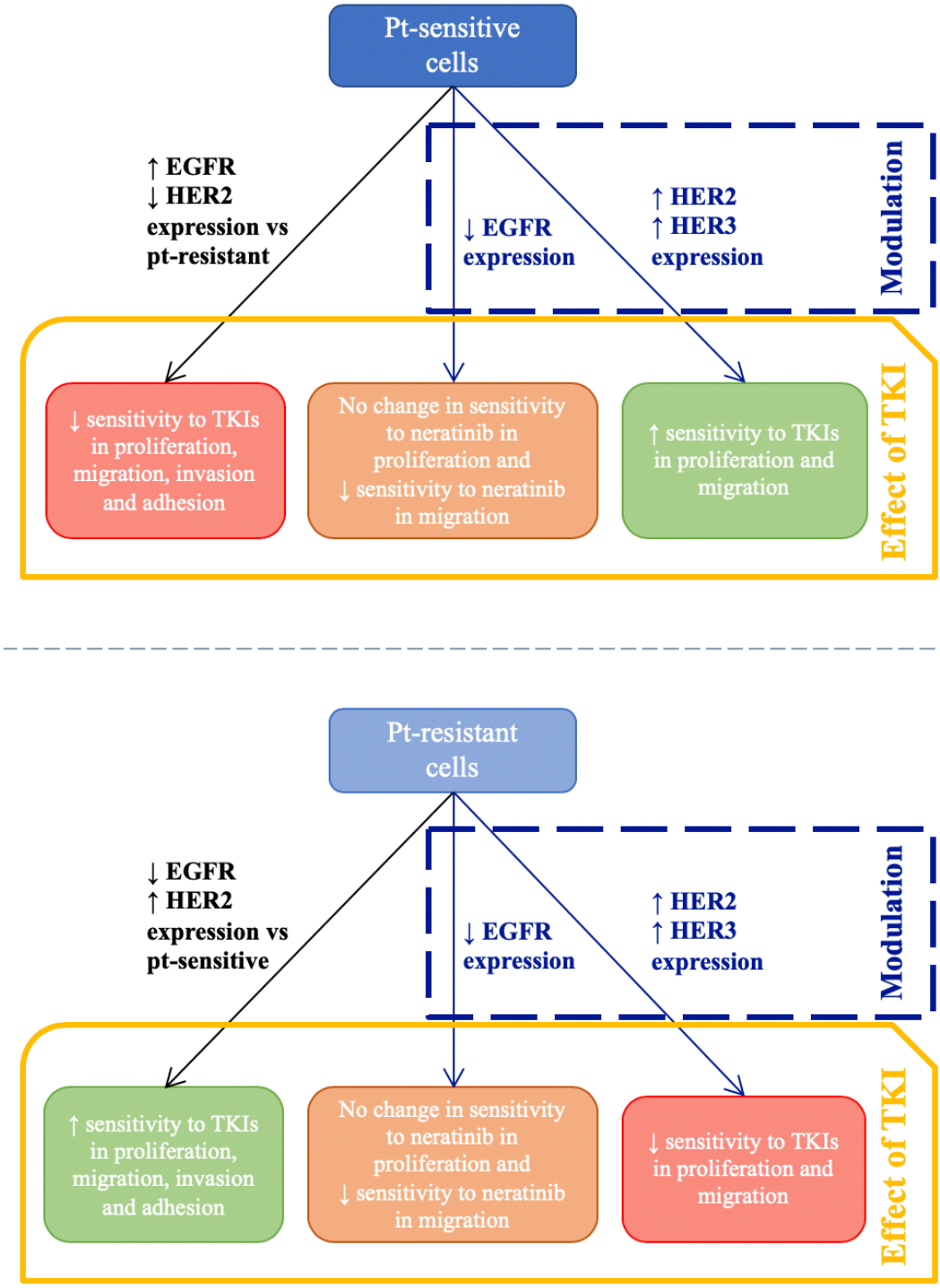


Figure 7.2: Summary of the effects of HER family modulation on the platinum-sensitive and platinum-resistant HGSOC cell lines

This flow chart summarises relevant results upon the decrease in EGFR expression, and the overexpression of HER2 and HER3, and presence of TKIs. The green boxes represent the favourable scenarios, the orange represent the neutral circumstances whilst the red boxes represent the unfavourable situations in the cellular effects of proliferation, migration and invasion.

7.1.4 Gene expression analysis identifies potential biomarkers for HER-targeted therapy

In concordance with western blot analysis, the gene expression analysis confirmed that HRG and neratinib modulated the PI3K and MAPK pathways in both PEA1 and PEA2 cell lines. Additionally, they also affected cell cycle processes. This finding was also supported by the Monks *et al.* dataset [446], whereby the addition of lapatinib to HGSOC cell lines modulated the PI3K and MAPK pathways.

This study then evaluated four HER-targeted therapy-modulated biomarkers, CEBPG, DDIT4L, RALGDS and SPRY2, which were co-expressed between the neratinib-treated cell lines and the trastuzumab-pertuzumab treated SKOV3 xenografts [232]. *CEBPG* and *RALGDS* gene and protein expressions were up-regulated upon HER-targeted treatment, while SPRY2 gene and protein expressions were down-regulated upon treatment. DDIT4L gene was down-regulated upon neratinib treatment, while the protein expression was up-regulated in the mAb-treated xenografts. Low levels of CEBPG and DDIT4L are often found in platinum-sensitive carcinoma, and associated with better PFS and OS, whilst higher expression of RALGDS and SPRY2 are found in platinum-sensitive disease and linked with favourable PFS and OS.

7.2 Future work

The cell line panel used in this study provided a clinically relevant model to study the effects of HER-targeted therapy and combination therapy, as the sensitivity and resistance to platinum-therapy originated naturally within patients. Such a series of paired cell lines provided a good opportunity to investigate the response to treatment on cell lines which underwent the processes of natural evolution, which would not have been possible from single-sample cell lines. These experiments were mostly done in 2D cell culture, as the approach is cost-effective, simple to set up and reproducible as it is commonly used. This is a clear limitation as this model does not reflect fully the cell-cell interactions, cell populations and structures and cell-extracellular matrix interactions that occur in patient tumours. Previous studies in breast cancer and colorectal cancer cell lines have found that the downstream activation of HER family is different between 2D and 3D cell models, whereby PI3K and MAPK pathways are

not phosphorylated in tandem in both models [500, 501]. Additionally, the response of 3D cells to trastuzumab was better than in 2D models, which further indicates that the extracellular matrix plays an important role in inhibitor response [500]. Thus, future investigations could utilize more 3D spheroids, tumour explants (*ex vivo*) or *in vivo* models. More recently, *ex vivo* explants in ovarian cancer have been successfully used as a model to study the chemosensitivity of carboplatin in a number of ovarian cancer tissues [502]. They offer cost-effective, rapid assays, which are easily reproducible and allow for more than one drug to be observed at the same time [502].

The promising results acquired from the platinum-resistant HGSOC in response to HER-targeted treatment create opportunities for further research. Further exploration is required to assess the sensitivity of other epithelial ovarian cancer types which are resistant to platinum-based chemotherapy from the outset, such as clear cell, mucinous and low-grade serous ovarian cancer subtypes. Areas to be explored might include growth, migration, and invasion, cell cycle flow cytometric analysis, as well as downstream effects potentiated by the inhibitors. The efficacy of the TKIs could be correlated with HER expression and compared with the potency in platinum-resistant cell lines. Biochemical techniques such as immunoprecipitation or proximity ligation assay could be used to assess receptor dimerization.

Previous studies have shown that treatment with gemcitabine followed by gefitinib produced more cytotoxic effects than the reverse sequence [503]. A limitation to this study was that it only assessed the effect of TKIs followed by cytotoxic agents. Hence, future studies could also investigate the role of an inverse sequencing of drugs and compare the difference in activities.

Resistant cell lines to TKIs could be derived from TKI-sensitive ovarian cancer cell lines. Investigations on these cell lines could include the response of these cells to cytotoxic agents and mAbs on various cellular functionalities. Furthermore, gene expression analysis could be performed upon TKI treatment to look at the differential gene expression between the two sets, and possibly, screen for probable biomarkers of sensitivity or resistance to TKI therapy.

The TKIs under investigation produced inhibitory effects even in the presence of EGF and HRG. Previous studies showed that the combination of trastuzumab and cisplatin in the presence of HRG-activated HER2 cells had an enhanced receptor chemo-sensitivity effect, even though HER2 was not over expressed [425]. Thus the combination of TKIs with mAbs or cytotoxic agents should be investigated in the presence of EGF or HRG, as EGF and HRG might modulate the sensitivity of cells to the combination agents. Further to this, the ability of TKIs to inhibit EGF- and HRG-modulated HER2 or HER3 transfected cell lines could also be investigated.

One of the points highlighted in Chapter 5 was that the expression level of HERs play an important role, even though this notion has not extrapolated to include mAbs. Future research could transfect cells with known amounts of the HERs, and use various different ratios of HER expression. The resultant cell lines could be investigated for their response to HER-targeted therapy, including mAbs and cytotoxic agents, to give a better understanding of the role of HERs as a biomarker for treatment sensitivity. Furthermore, the EGFR knockdown of the other cell lines could also form part of further research and the effects of the other four TKIs could also be assessed in addition to neratinib.

Gene expression analysis was only performed on two cell lines, using only neratinib at a single time point. Neratinib was used for its novelty, but there are no patient gene expression data upon neratinib treatment available as far as we are aware that could be compared. Thus, potential further studies could look at more HGSOC platinum-sensitive and platinum-resistant cell lines to validate the results, and also other ovarian cancer cell line subtypes to compare and contrast findings in the absence of patient data. The therapy-induced biomarkers could also be validated using cell-line samples treated with other HER-targeted therapy on western blots or techniques such as reverse phase protein arrays. Additionally, other TKIs could be evaluated to compare the genes and pathways that are mostly affected upon TKI treatment and get a better understanding of the molecular characteristics of tumours that are likely to respond to these agents.

7.3 In summary

These results show that the broad-spectrum TKIs may be useful in HGSOC patients, irrespective of whether they are sensitive or resistant to platinum therapy as they possessed anti-tumour activity across multiple types of cellular functionality, including proliferation, migration and invasion. The presence of EGF and HRG did not diminish the inhibitory effects of the TKIs on these cell lines. Additionally, the importance of the expression levels of the HER family of receptors has been highlighted, and a main inference was the significance of the ratio of HER2 and HER3 expression for optimal response to TKIs. This study has also identified four potential HER-targeted therapy-induced biomarkers (CEBPG, DDIT4L, RALGDS and SPRY2), whose expression changed upon neratinib treatment. The expression of these proteins has been assessed in xenografts treated with mAbs, and the results generally reflected the gene expression changes.

In conclusion, this present study contributes to our understanding of the involvement of the HER family towards the activity of HER-targeted TKIs in HGOSC. The expression of the HER family could be important as a biomarker for response to therapy. Broad spectrum TKIs have shown great potential, even in platinum-resistant HGSOC cell lines.

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Appendix 1: Supplementary Figures to Chapter 5

In PEO1 and PEO4 cell line transfects, HRG produced the highest stimulation in p-HER2, whilst EGF produced the highest stimulation on p-ERK. All TKIs were able to inhibit the stimulation, especially sapitinib and lapatinib.

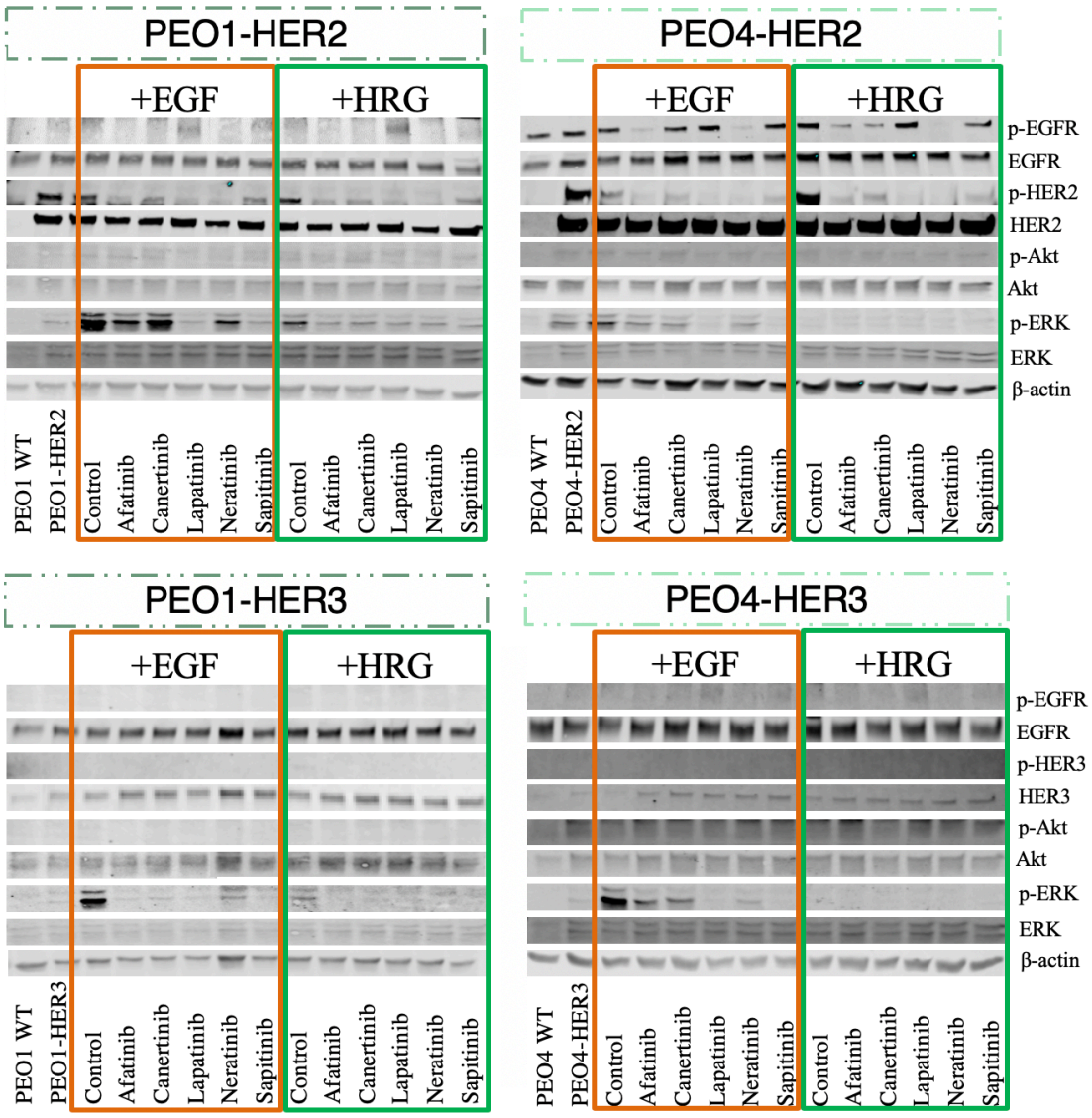

Figure 7.3: HER pathway and downstream signalling proteins for PEO1 HER2 or HER3 and PEO4 HER2 or HER3 modulated cell lines when EGF/HRG with or without TKIs were added

TKIs were added at clinically relevant concentrations for 24 h; 30 min before lysate collection, 1 ng/mL EGF or HRG were added to the cells. The experiment was repeated once and the loading controls are a representation to the other membranes.

In PEO23 cell line transfects, HRG produced the highest stimulation in p-HER2, whilst EGF produced the highest stimulation on p-ERK in both PEO14 and PEO23 cell line transfects. All TKIs were able to inhibit the stimulation, especially sapitinib and lapatinib.

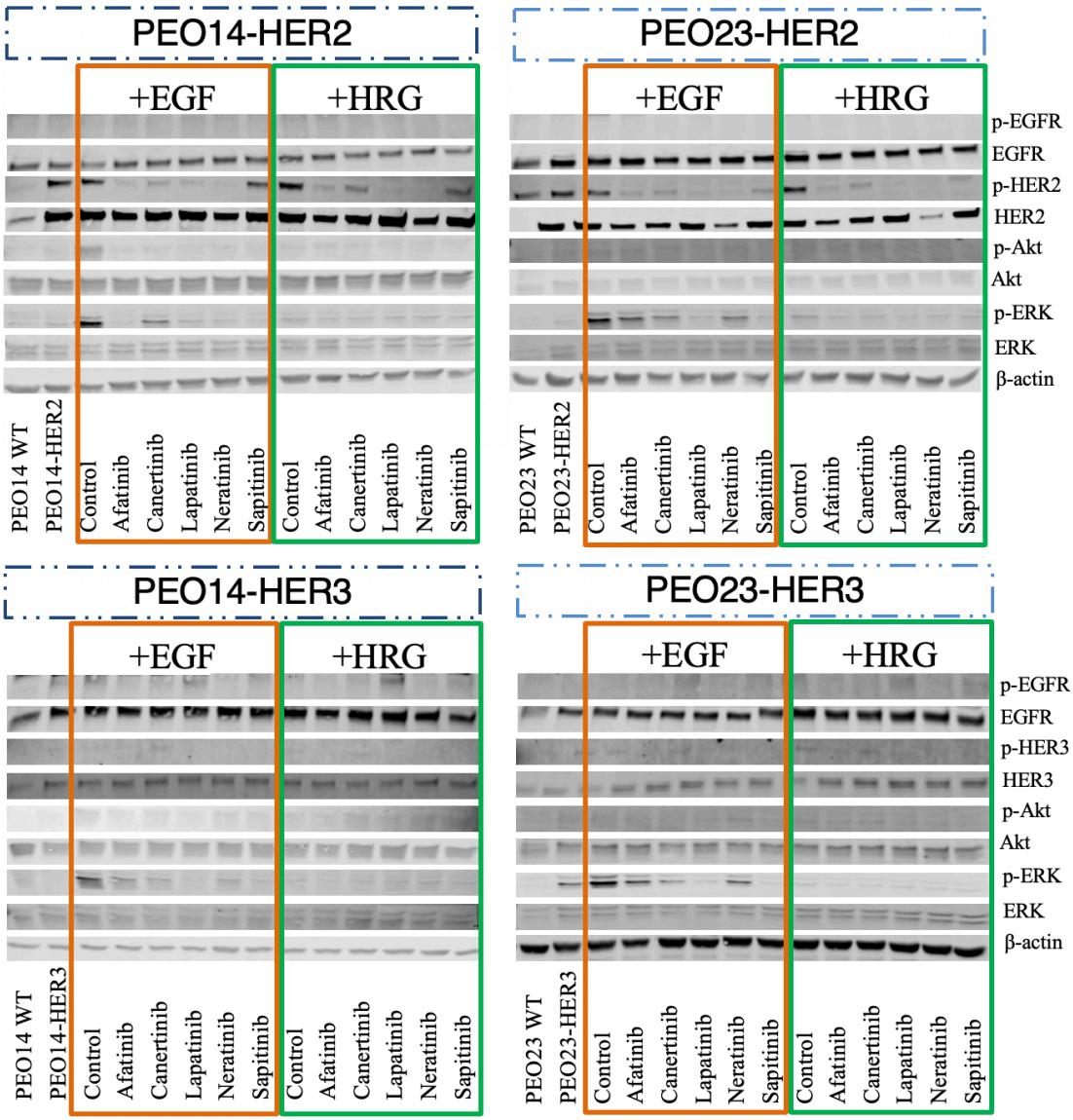

Figure 7.4: HER pathway and downstream signalling proteins for PEO14 HER2 or HER3 and PEO23 HER2 or HER3 modulated cell lines when EGF/HRG with or without TKIs were added

TKIs were added at clinically relevant concentrations for 24 h; 30 min before lysate collection, 1 ng/mL EGF or HRG were added to the cells. The experiment was repeated once and the loading controls are a representation to the other membranes.

Appendix 2: Quality Control of RNAseq samples

Sample	RIN	Bases	≥ Q20	Total Reads	Aligned Reads	Mean Read length (bp)
PEA1 C (1)	9.9	942,706,443	769,210,714	6,975,972	6,907,793	135
PEA1 N (1)	9.9	906,201,298	742,948,365	6,910,986	6,849,133	131
PEA1 H (1)	9.9	926,879,722	762,335,982	6,960,239	6,906,593	133
PEA1 NH (1)	9.9	894,906,279	735,365,168	6, 651,204	6,604,386	135
PEA2 C (1)	9.8	1,244,237,192	1,016,741,095	9,604,452	9,505,304	130
PEA2 N (1)	9.6	719,272,762	588,884,424	5,565,713	5,513,034	129
PEA2 H (1)	9.8	936,086,908	764,143,495	7,003,905	6,935,004	134
PEA2 NH (1)	9.7	830,953,660	681,740,628	6,575,713	6,512,708	126
PEA1 C (2)	9.8	939,018,277	763,897,551	6,781,433	6,726,471	138
PEA1 N (2)	9.7	996,540,530	816,869,081	7,721,759	7,654,662	129
PEA1 H (2)	9.8	831,208,597	680,490,573	6,339,800	6,290,089	131
PEA1 NH (2)	9.2	978,635,527	803,797,438	7,126,631	7,078,638	137
PEA2 C (2)	9.1	539,412,037	443,277,531	4,160,791	4,124,122	129
PEA2 N (2)	8.1	420,972,836	346,970,226	3,147,622	3,120,803	133
PEA2 H (2)	8.9	596,363,800	490,937,897	4,325,195	4,290,092	137
PEA2 NH (2)	8.7	532,337,540	438,048,162	4,139,080	4,101,524	128
PEA1 C (3)	9.8	693,285,946	572,264,352	5,312,283	5,266,940	130
PEA1 N (3)	9.8	598,314,451	492,567,143	4,412,104	4,377,633	135
PEA1 H (3)	9.9	773,131,593	634,586,202	5,871,867	5,817,549	131
PEA1 NH (3)	9.9	3,813,922,939	3,129,533,054	28,490,483	28,183,048	133
PEA2 C (3)	9.7	352,061,071	288,549,979	2,896,080	2,859,762	121
PEA2 N (3)	9.5	743,832,034	612,399,144	5,749,030	5,702,730	129
PEA2 H (3)	9.6	587,886,834	484,779,249	4,414,947	4,375,783	133
PEA2 NH (3)	9.3	514,249,317	423,111,990	3,808,012	3,768,616	135

Figure 7.5: Quality control data of the RNAseq samples

The Agilent QC report was issued by Edinburgh Clinical Research Facility, University of Edinburgh. Where C = control, N = neratinib, H= heregulin, RIN = RNA integrity number, Q = quality score.

Appendix 3: Gene Lists to Chapter 6

Genes related to Section 6.1

Term	PValue	Pvalue (log)	Genes	Fold Enrichment	Bonferroni	Benjamini	FDR
ECM-receptor interaction	2E-04	4E+00	TNC, ITGA11, ITGA4, ITGB3, COL4A5, HMMR, LAMA2, ITGB8, COL27A1, COL6A3, COL6A1, RELN, SV2A, COL1A1, THBS1, SPP1, FN1	2.9	0.1	0.1	0.3
Focal adhesion	3E-04	3E+00	PDGFB, TLN2, PGF, TNC, ITGA11, ITGB3, MYL9, ITGB8, COL27A1, BCL2, COL6A3, COL6A1, THBS1, SPP1, FN1, MYLK4, MET, MAPK10, ITGA4, FLNC, BIRC3, COL4A5, LAMA2, CCND3, PDGFRB, RELN, COL1A1, CRK, MYLK	2.1	0.1	0.0	0.4
PI3K-Akt signalling pathway	1E-03	3E+00	FGFR2, FGFR4, PDGFB, PGF, TNC, LPAR4, ITGA11, TLR2, LPAR2, ITGB3, IL7R, ITGB8, BCL2, COL27A1, COL6A3, COL6A1, PIK3AP1, GNG2, GNG4, THBS1, MYC, CHUK, SPP1, IL6, IL7, MET, CREB5, CDK6, ITGA4, PCK2, IRS1, COL4A5, LAMA2, CDKN1A, CCND3, PDGFRB, RELN, COL1A1	1.7	0.3	0.1	1.5
TNF signalling pathway	6E-03	2E+00	ICAM1, CFLAR, IL6, MMP9, CREB5, CX3CL1, MAPK10, BIRC3, CCL5, MMP14, VCAM1, TNFRSF1B, MAPK12, MAPK13, MAP2K6, CHUK	2.2	0.8	0.2	7.4
Transcriptional mis-regulation	2E-02	2E+00	IL1R2, NFKBIZ, TMPRSS2, IL6, ERG, MMP9, MET, PAX5, CD40, AFF1, HMGA2, MYCN, CDKN1A, ETV1, HIST1H3D, PBX1, RUNX2, HPGD, MYC	1.7	1.0	0.2	22.3
Cell adhesion molecules (CAMs)	3E-02	1E+00	CLDN16, ICAM1, NRXN3, CLDN3, CLDN6, NLGN1, CD40, ITGA4, CLDN11, HLA-DMB, VCAM1, ITGB8, CLDN1, CLDN2, CNTNAP1, JAM2, NEGR1	1.7	1.0	0.3	35.0
Wnt signalling pathway	0.01	1.84	WNT5A, FZD9, WNT10A, NKD1, WNT3A, MMP7, FZD3, PORCN, WNT7B, WNT4, DKK1, SFRP1, CAMK2B, SOX17, WNT9A, WNT7A	2.0	1.0	0.3	17.3
MAPK signalling pathway	0.02	1.65	FGF18, CACNA2D1, HRAS, TNF, CACNG8, MKNK2, PTPRR, CACNG4, HSPA1A, FGF12, MECOM, CACNA2D3, CACNA2D2, MAP3K6, KRAS, RPS6KA1, RRAS2, MAPT, PDGFRA, CACNA1H, MAPK8IP1, DUSP9, DUSP8, IL1A	1.6	1.0	0.4	25.5
ECM-receptor interaction	0.07	1.18	COL4A4, COL4A3, LAMB3, ITGA6, ITGB7, COL1A2, ITGB4, AGRN, COL11A1, COL5A1	2.0	1.0	0.6	59.3

Table 7.1: The list of genes for the pathways related to the most differently expressed genes between the PEA1 and PEA2 cell line pair.
Pathways related to the differently expressed genes and the –log10 p-value of each pathway analysed using DAVID online resource. The blue entries represent the pathways that are lower expressed in PEA1 cell line with respect to PEA2 cell line, while the red entries represent the pathways that are more highly expressed in PEA1 cell lines versus PEA2 cell line.

Term	PValue	Genes	Fold Enrichment	Bonferroni	Benjamini	FDR
Cell cycle	0.004	ACVRL1, ENPP1, CCDC85B, FHL1, SIPA1, FBP1, NPR1, GJA1, CAPRIN2, NOV, HNF4A, SERPINE2, SFRP1, CDKN2D, CDA, SOX17, MT3, MYC, FOXL2, CDKN2A	2.2	1.0	0.4	7.4
Cell migration	0.013	HRAS, RET, BMP2, LYN, PODXL, FAM110C, EDN1, KIT, MIEN1, CCL26, CDH13, SEMA6B, ITGA6, F3, RRAS2, PDGFRA, SEMA4B, SEMA3B, CTSH, PIK3R1, F2R, MET, ENG	1.8	1.0	0.6	21.8
Cell adhesion	0.011	SCN1B, PCDHA2, AMTN, GNE, PCDHB15, ITGB4, BCAM, SPOCK1, L1CAM, EDIL3, APLP1, RGMB, WISP2, LAMB3, AGGF1, ITGB7, DGCR6, MSLN, CNTNAP3, COL11A1, BOC, COL4A3, HAPLN3, SELL, ADAM23, EFNB1, PODXL, EFNB2, PCDH10, NID2, PTPRU, EMILIN2, COL5A1, ARVCF, CDH13, EPHA4, ITGA6, CDH16, CD34, CDON, NLGN4X, ADAM22, PARVA, CLDN7	1.5	1.0	0.6	18.5
HER signalling pathway	0.009	HRAS, KRAS, ERBB4, ERBB3, ERBB2, PTPRR, PIK3R1, MATK	3.3	1.0	0.5	15.6
Wnt signalling pathway	0.006	FZD9, WNT5A, WNT3A, FZD3, PTPRU, PORCN, WNT7B, WNT4, SFRP1, HOXB9, SOX17, WNT9A, WNT7A	2.5	1.0	0.5	10.6

Table 7.2: The list of genes for the biologic processes related to the most differently expressed genes between the PEA1 and PEA2 cell line pair.

Biological processes related to the differently expressed genes and the p-value analysed using DAVID online resource. The blue entries represent the processes that are lower expressed in PEA1 cell line with respect to PEA2 cell line, while the red entries represent the pathways that are more highly expressed in PEA1 cell lines versus PEA2 cell line.

1. **PEA1**

Term	PValue	Genes	Fold Enrichment	Bonferroni	Benjamini	FDR
MAPK signalling pathway	0.032	DUSP5, PPP5D1, PLA2G4A, DUSP2, NTF3, CACNG8, JUN, RASGRP1, CACNG6, DUSP16, TGFB3, MAPK8IP1, FGF1, NGF, DUSP6	1.85	1.00	0.75	33.45

Table 7.3: Genes of the pathways represented by the differently expressed genes in PEA1 cells across different treatment groups.

The differently expressed genes in the different treatment. The analysis was carried out using Rank Products with 5% FDR. The significant differently expressed genes within each group were defined in accordance with the DAVID online resource.

2. **PEA2**

Term	PValue	Genes	Fold Enrichment	Bonferroni	Benjamini	FDR
TNF signalling pathway	0.006	LIF, ATF4, TNF, MAPK12, CXCL2, PIK3R3	5.1	0.7	0.7	7.2
FoxO signalling pathway	0.015	TNFSF10, CCND1, SGK2, MAPK12, PCK2, PIK3R3	4.1	0.9	0.6	17.1
Jak-STAT signalling pathway	0.021	LIF, CCND1, PIM1, PIK3R3, MYC, GHR	3.7	1.0	0.5	22.6
PI3K-Akt signalling pathway	0.033	ATF4, CCND1, SGK2, NR4A1, PCK2, PIK3R3, MYC, EPHA2, GHR, CCNE2, LAMC3, ATF6B, TP53, KITLG, JAK3, ITGB3, NGF, ITGA2B	2.4	1.0	0.6	34.1
MAPK signalling pathway	0.057	DUSP5, ATF4, TNF, MAPK12, NR4A1, MYC, DUSP6, MEF2C, DUSP2, HSPA2, TP53, PLA2G4C, DDIT3, NGF	2.5	1.0	0.6	51.4
P53 signalling pathway	0.036	CCNE2, TP53, SESN2, CCNG2	5.4	1.0	0.6	35.9

Table 7.4: Genes of the pathways represented by the differently expressed genes in PEA2 cells across different treatment groups.

The differently expressed genes in the different treatment. The analysis was carried out using Rank Products with 5% FDR. The significant differently expressed genes within each group were defined in accordance with the DAVID online resource.

3. Monks et al. dataset [438] versus my dataset

Term	PValue	PValue (log)	Genes	Fold Enrichment	Bonferroni	Benjamini	FDR
TNF signalling pathway	4.7E-05	4.33	LIF, FOS, CCL20, EDN1, CXCL2, PIK3R3, PIK3R1, JUNB, PIK3R2	6.73	0.01	0.01	0.1
FoxO signalling pathway	1.3E-03	2.90	SGK1, TNFSF10, PLK3, PCK2, PIK3R3, CCNG2, PIK3R1, PIK3R2	4.78	0.19	0.07	1.5
mTOR signalling pathway	5.5E-03	2.26	ULK1, PIK3R3, PIK3R1, DDIT4, PIK3R2	6.90	0.61	0.11	6.6
HIF-1 signalling pathway	6.5E-03	2.19	EDN1, VEGFA, ENO3, PIK3R3, PIK3R1, PIK3R2	5.00	0.67	0.12	7.6
MAPK signalling pathway	1.3E-02	1.90	DUSP5, DUSP4, FOS, HSPA2, DUSP10, HSPA1A, MYC, DDIT3, DUSP6	2.85	0.88	0.14	14.2
Rap1 signalling pathway	1.5E-02	1.83	ADORA2B, VEGFA, PRKCI, CDH1, ARAP3, PIK3R3, PIK3R1, PIK3R2	3.05	0.92	0.15	16.6
HER signalling pathway	2.2E-02	1.65	HBEGF, PIK3R3, MYC, PIK3R1, PIK3R2	4.60	0.98	0.17	24.0
PI3K-Akt signalling pathway	2.6E-02	1.59	SGK1, ITGB8, VEGFA, COL2A1, PCK2, PIK3R3, MYC, PIK3R1, DDIT4, PIK3R2	2.32	0.99	0.16	27.3
VEGF signalling pathway	3.9E-02	1.41	VEGFA, PIK3R3, PIK3R1, PIK3R2	5.25	1.00	0.19	38.6
Apoptosis	4.1E-02	1.39	TNFSF10, PIK3R3, PIK3R1, PIK3R2	5.16	1.00	0.19	39.9

Table 7.5: Genes of the pathways represented by the overlapping differently expressed genes between lapatinib-treated and neratinib-treated HGSOC cell lines.

The differently expressed genes in dataset GSE116436 [438] were evaluated using FDR of 5%. The untreated control data was compared against 1 μM lapatinib treatment for 6 h. The genes taken into consideration for pathway evaluation were the genes which had at least one lapatinib-treated and one neratinib-treated cell line involvement. The pathways were analysed using DAVID online resource and the most significant ones are shown.

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REVIEW

Human epidermal growth factor receptor targeted inhibitors for the treatment of ovarian cancer

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ABSTRACT Ovarian cancer is the second most lethal gynecological cancer worldwide and while most patients respond to initial therapy, they often relapse with resistant disease. Human epidermal growth factor receptors (especially HER1/EGFR and HER2/ERBB2) are involved in disease progression; hence, strategies to inhibit their action could prove advantageous in ovarian cancer patients, especially in patients resistant to first line therapy. Monoclonal antibodies and tyrosine kinase inhibitors are two classes of drugs that act on these receptors. They have demonstrated valuable antitumor activity in multiple cancers and their possible use in ovarian cancer continues to be studied. In this review, we discuss the human epidermal growth factor receptor family; review emerging clinical studies on monoclonal antibodies and tyrosine kinase inhibitors targeting these receptors in ovarian cancer patients; and propose future research possibilities in this area.

KEYWORDS Tyrosine kinase; monoclonal antibodies; ovarian cancer; inhibitors; HER; receptors

Introduction

Ovarian cancer is the eighth most lethal type of cancer in women worldwide, with over 184,000 fatalities reported in 2018[1]. It has a poor prognosis and is usually diagnosed at late stage, due to lack of specific diagnostic biomarkers and relatively non-specific symptoms. Current diagnostic tests for ovarian cancer include the CA-125 blood test coupled with abdominal/pelvic ultrasound and computerized tomography (CT) scan. First line treatment of ovarian cancer is optimal debulking of macroscopic disease, generally followed by chemotherapy with carboplatin alone or in combination with paclitaxel[2,3].

The most common type of ovarian cancer is epithelial ovarian cancer (EOC), which is divided into 5 main subtypes, with differing histological, molecular, and genetic characteristics[3,4]. The most common subtype is high grade serous ovarian cancer (HGSOC) which accounts for approximately 70% of the cases, with clear cell (10%), endometrioid (10%), mucinous (< 5%), and low grade serous cancer (< 5%) comprising the other significant subtypes[3,4]. Recent investigations into the pathogenesis of ovarian cancer showed that it primarily originates from different parts of the female reproductive system and involves cellular migration to the ovaries. HGSOC is identified to originate from the distal fallopian tube, endometrioid and clear cell cancers arise from the endometrium, while low grade serous cancer might progress from serous cystadenoma and serous borderline tumors[5].

Multiple drugs have been tested and approved for ovarian cancer although the response rate for second line therapy is only 10%–35% and different ovarian cancer subtypes respond differently to drug treatment. HGSOC patients usually respond well to initial platinum-based therapy, given their BRCA and p53 mutations[7]. However, these patients often present with resistance to initial therapy after a few months.

There is increasing interest in the potential use of targeted inhibitors for the treatment of ovarian cancer. This review seeks to overview the current clinical and preclinical status of human epidermal growth factor receptor (HER) targeted therapy in ovarian cancer, with special emphasis on tyrosine kinase inhibitors (TKIs).

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Human epidermal growth factor receptors (HERs)

The HER family has been associated with the progression of several cancers including breast, lung and colon cancer[8]. In ovarian cancer, amplification, and/or high expression of epidermal growth factor receptor (EGFR), HER2 and HER3 receptors have been implicated in the progression and prognosis of the disease[9-12].

The HER family of receptors [also known as erythroblastic leukemia viral oncogene (erbB) family] are present on the cell surface as monomers, in the absence of ligand activation. There are four members in this family, EGFR (HER1/erbB1), HER2 (neu/erbB2), HER3 (erbB3) and HER4 (erbB4) (**Figure 1**). With the exception of HER2, ligands bind to their extracellular domain and form homo- or heterodimers with other members of the family, preferentially with HER2, since it has the most favorable kinase activity and exists in an activated form[13]. HER ligands are divided into three groups; those which bind specifically to EGFR (epidermal growth factor, amphiregulin and transforming growth factor-α), those conferring dual specificity to EGFR and HER4 (betacellulin, heparin-binding EGF, and epiregulin), and those which bind to HER3 and HER4 (neuregulins/heregulins)[14].

Upon receptor dimerization, multiple downstream pathways are activated, which regulate cell proliferation, differentiation, angiogenesis, survival, and cellular metabolism amongst other functions. Heterodimerization allows for a myriad of phosphotyrosine residues to bind,

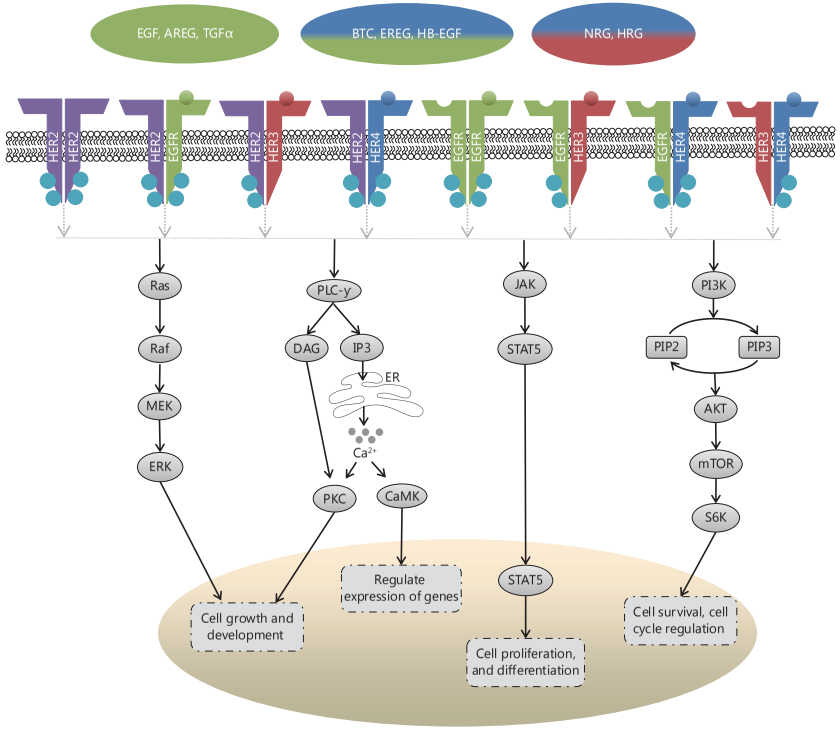


Figure 1 Scheme illustrating the downstream signaling of the HER pathway, chiefly the mitogen activated protein kinase (MAPK)/ERK pathway, the phospholipase Cγ (PLCγ) pathway, the signal transducer and activation of transcription (STAT) pathway, and the phosphoinosidyl-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway; where ER=Endoplasmic Reticulum, Ca^{2+}=Calcium ions

which in turn increases the possibilities for signaling pathways[15]. These pathways (**Figure 1**) include the mitogen activated protein kinase (MAPK)/ERK pathway, which regulates the growth and development of cells, the signal transducer and activation of transcription (STAT) pathway, which governs cell proliferation and differentiation, the phosphoinosidyl-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, which regulates cell survival and metabolism, and the phospholipase Cγ (PLCγ) pathway, which controls calcium-dependent actions[13,16,17]. In tumorigenesis, mutations within the components of these pathways can cause cancer cells to acquire certain aptitudes, including impartiality to proliferation signals, circumvention of apoptosis, insensitivity to growth inhibitory signals, augmented replicative potential and the capability to metastasize[18].

EGFR (HER1) is a receptor tyrosine kinase comprising an extracellular ligand-binding domain of 622 residues, a 23 residue transmembrane domain, and a large, 522-residue intracellular domain[15]. EGFR is normally weakly expressed in the ovaries, however, several studies have found that EGFR is highly expressed in ovarian cancer. Immunohistochemical studies have indicated that 30%–70% of ovarian cancers have increased EGFR expression[19-21]. High expression of EGFR is associated with poor, progression-free survival (PFS), advanced tumor grade, greater residual tumor mass and rapid proliferation[9,10,12]. It has also been suggested that high EGFR expression in the tumor stroma is associated with aggressive clinical conditions and outcome and EGFR upregulation in fibroblasts is associated with growth and migratory abilities of ovarian cancer cells[22].

HER2 is overexpressed in approximately 6%–30% of ovarian cancer patients[10,23] and is initially associated with DNA amplification and poor prognosis[24]. Overexpression is often detected in the mucinous (19%)[25] and clear cell (14%)[26] subtypes. However, even some serous (3%) and endometrioid ovarian cancers (2%) have HER2 overexpression[27]. Several studies have associated overexpression of HER2 with poor prognosis[10,28].

HER3 is expressed as a full-length receptor on the cell surface, in parallel with truncated intracellular isoforms. However, the activity of the latter is not well defined[29,30]. HER3 lacks tyrosine kinase activity, hence it has to be transphosphorylated by other HER members to promote cell signaling[15]. HER3 is more frequently expressed in ovarian cancer (30%–80%) than EGFR and HER2[31] and is more common amongst borderline and early-stage lesions[32]. Among the dimerization possibilities between these proteins, the most potent signaling complex is generated when HER3 heterodimerizes with HER2[33]. Increased HER3 expression has been associated with poor clinical outcome and the average survival time for patients with low HER3 expression was 3.3 years, in contrast to 1.8 years for patients with high HER3 expression[11]. Studies in various cancers have shown that when HER3 and MET are co-expressed, they are often associated with either response or resistance to therapy[34-36]. Other studies have shown that high expression of HER3 might lead to HER3-PI3K-Akt signaling cascade in doxorubicin and cisplatin treated ovarian cancer, which often results in resistance to therapy[37,38].

HER4 is the least understood receptor of the HER family. It occurs as a spliced isoform, often being processed further by enzymes into a soluble intracellular domain, which can disperse to the cell cytoplasm or nucleus[39]. In breast cancer, nuclear localization of the intracellular domain in combination with estrogen expression predicted worse clinical outcomes compared to membrane HER4 and estrogen[40]. There are conflicting views about the expression of HER4 in ovarian cancer, with earlier reports suggesting either decreased or lack of expression of the receptor[41], while more recent studies suggest an increased expression of HER4 in malignant tissues compared to normal tissues[31,42,43]. Although the implication of HER4 expression in ovarian cancer is unclear, two studies found a possible correlation between HER4 expression and resistance of serous ovarian cancer to chemotherapy[42,44].

Monoclonal antibodies

HER-targeted monoclonal antibodies (mABs), such as trastuzumab (Herceptin®) and pertuzumab (Perjeta®) are recombinant humanized mABs, inhibiting HER2 extracellularly with differing modes of action (**Figure 2**). These agents have shown favorable results in HER2 positive cancers, especially HER2-positive breast cancer, where they are well established as standard therapy. More recently, trastuzumab-emtansine antibody-drug conjugate has been developed as another option for trastuzumab-resistant disease. Preclinical *in vitro* and *in vivo* studies and clinical trials have been focusing on the activity of these mABs in ovarian cancer, especially in selective subtypes, particularly mucinous cancers, which have HER2 amplification and overexpression[45,46].

Trastuzumab

Trastuzumab binds to the juxtamembrane region of the extracellular domain of HER2, inhibiting cleavage of the

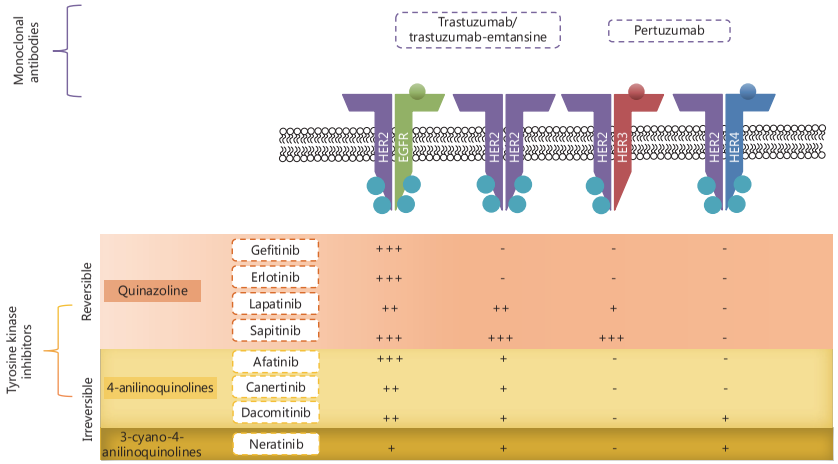


Figure 2 Site of action of the various TKIs and the relative potency towards the receptor; where +++ is very potent, ++ is potent, + is mildly potent and – is generally not active.

extracellular domain, obstructing HER2 homodimerization as well as inducing antibody dependent cell-mediated cytotoxicity[47,48]. Trastuzumab requires HER2 overexpression for it to be effective[48,49]. The success of trastuzumab in clinical trials[49-51] has led to its clinical approval in metastatic breast cancer overexpressing HER2, as monotherapy or in combination with paclitaxel depending on whether the patients previously received chemotherapy regimens for their metastatic disease[52]. A phase II clinical trial of trastuzumab involving 41 eligible ovarian cancer patients with HER2 overexpression demonstrated an overall response rate (ORR) of 7.3%, which included one complete and two partial responses. The median PFS was two months[53].

Pertuzumab

Pertuzumab acts by blocking the dimerization domain of HER2, thus inhibiting HER2 heterodimerization[48]. Unlike trastuzumab, it does not require HER2 overexpression to confer its inhibitory effects[54]. Pertuzumab is used to treat HER2-positive metastatic breast cancer patients who have not been previously exposed to anti-HER2 therapy or chemotherapy for metastatic disease and is also used for the neoadjuvant treatment of HER2-positive early stage breast cancer[55]. In a phase III clinical trial for breast cancer (CLEOPATRA), the combination of trastuzumab and pertuzumab along with docletaxel, showed additional benefit compared to monotherapy, which has subsequently led to the approval of this combination for HER2-positive metastatic breast cancer[56]. A randomized phase II clinical trial with pertuzumab showed better PFS (5.3 months) in low HER3 expressed, platinum-resistant ovarian cancer patients and hence it was assessed in a phase III clinical trial (PENELOPE) in platinum-resistant ovarian cancer patients with low expression of HER3[57,58]. In the PENELOPE trial, patients who received pertuzumab with chemotherapy showed a PFS of 4.3 months and an ORR of 13.1%, when compared to the PFS of 2.6 months and ORR of 8.7% in patients who received placebo with chemotherapy[58]. In an *in vivo* setting using ovarian cancer xenograft mouse models, our group has demonstrated that the combination of pertuzumab and trastuzumab produces prolonged growth inhibition, when compared to either antibody used as a single agent[59]. Furthermore, another preclinical study has suggested that trastuzumab could also augment sensitivity to endocrine therapy in ERα-positive ovarian cancer[60].

Trastuzumab-emtansine

Trastuzumab-emtansine (T-DM1, Kadcyla®), is a HER2 targeted mAB conjugated to a microtubule inhibitor (emtansine). T-DM1 acts by binding to HER2, triggering the endocytosis of the HER2-T-DM1 complex. Once in the cytoplasm, DM1 is released from the complex, which inhibits

microtubule assembly, leading to cell death[61]. In a phase III clinical trial for breast cancer, T-DM1 treated cohort showed a superior clinical outcome (PFS 9.6 months) compared to patients who received lapatinib with capecitabine (6.4 months)[62]. It was later approved as monotherapy for HER2-positive metastatic breast cancer, which is resistant to trastuzumab treatment[63]. In a recent *in vivo* study by Menderes et al.[64], T-DM1 demonstrated significant tumor growth inhibition against HER2 overexpressing ovarian cancer primary cell lines compared to either pertuzumab or trastuzumab alone or a combination of both. It also reduced tumor growth and improved survival in xenograft models[64-66]. This agent was also shown to have similar antibody-dependent, cell-mediated cytotoxicity as pertuzumab, trastuzumab and their combination[64]. Another study demonstrated their excellent inhibitory activity against both subcutaneous and intraperitoneal growth of the SKOV3 in an ovarian cancer xenograft model[67].

TKIs

TKIs are small drug molecules that inhibit tyrosine kinases. Tyrosine kinases include the HER family, vascular endothelial growth factor receptors (VEGF), platelet-derived growth factor receptors (PDGFR), and also non-receptor tyrosine kinases BCR-ABL and KIT[68,69]. Tyrosine kinases are enzymes that catalyze the transfer of phosphate from adenosine triphosphate (ATP) onto target proteins to elicit a response. There are three types of TKIs. Most small molecule TKIs are type I, which compete with ATP by binding to ATP binding sites on the active conformation of the receptors, thereby interfering with the action of tyrosine kinases[69]. Type II TKIs bind to the inactive conformation of a kinase, while type III allosteric inhibitors bind to sites distant from the active site[70]. To date, a few TKIs have been evaluated in ovarian cancer patients that are described below (**Table 1**, **Figure 2**). These include the first generation EGFR inhibitors, gefitinib and erlotinib, which have shown clinical efficacy against mutant EGFR lung cancer. Since, a resistance mutation develops frequently at T790M upon treatment, covalent irreversible second generation TKIs were developed. These consist of afatinib and neratinib that are active against this mutation. Other HER inhibitors were developed with broader inhibitory activity across multiple HER family members (pan-inhibitors) and these include lapatinib and canertinib as early developed inhibitors, followed by neratinib, sapitinib, and dacomitinib. Finally, multi-targeted TKIs that target the HER family among other targets (e.g., PDGFR, VEGFR, etc.) include vandetanib and leflunomide.

Reversible inhibitors

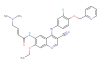
Gefitinib

Gefitinib (Iressa®, ZD-1839) is a type I quinazoline derivative TKI, reversibly selective for EGFR. Gefitinib is marketed for monotherapy of locally advanced or metastatic NSCLC and is most effective in cancers with mutation or increased EGFR copy number[81]. When evaluated in an ovarian cancer phase II trial, gefitinib showed a limited response rate in platinum pre-treated ovarian cancer patients, with only one out of twenty-seven patients having an ORR of 4%[82]. However, this cancer was the one in this cohort harboring a mutation in the catalytic region of EGFR, consistent with data for NSCLC. Another phase II clinical trial combining gefitinib with tamoxifen in refractory or resistant ovarian cancer patients, did not demonstrate any advantageous tumor responses with median time-to-progression being 58 days[83]. Preclinical studies in ovarian cancer demonstrate that combining gefitinib with cisplatin, increases the efficacy of cisplatin, mainly due to the inhibition of downstream EGFR signaling and blocking DNA repair mechanisms[84].

Erlotinib

Erlotinib (Tarceva®, OSI-774) is a quinazoline derivative, which reversibly inhibits EGFR. It is indicated as a first line therapy for NSCLC and in combination with gemcitabine for pancreatic cancer. Erlotinib has shown enhancement of PFS compared to chemotherapy (13.1 months vs. 4.6 months, respectively) in chemotherapy-naive NSCLC patients, harboring EGFR mutations[85]. An *in vivo* study in ovarian cancer expressing high EGFR demonstrated that a combination of erlotinib with olaparib, a PARP inhibitor, had a greater tumor suppressive effect than monotherapy[86]. A phase II study combining erlotinib with carboplatin showed that this combination was more effective in ovarian cancer patients with platinum-sensitive disease compared to platinum-resistant disease with 57% and 7% objective response rates, respectively. However, the contribution of erlotinib in this combination is unclear[87]. Another phase II study assessing the pathologic complete response (pCR) of ovarian cancer patients administered with a combination of carboplatin, paclitaxel, and erlotinib, resulted in pCR of around 30%, which was not an improvement when compared to previous results[88]. Continuous infusion of topotecan with erlotinib was studied in a phase II trial, where only 1 out of 6 patients showed a satisfactory partial response[89]. An exploratory phase II clinical trial involving bevacizumab and erlotinib determined that high levels of VEGF-A caused bevacizumab resistance, while erlotinib did not seem to contribute to the efficacy of the combination[90]. A randomized phase III study evaluated the efficacy of administering erlotinib to patients with ovarian cancer after first line chemotherapy. The median PFS in patients receiving erlotinib and placebo was 12.7 and 12.4 months, respectively, concluding that erlotinib does not render pre-treated ovarian cancer patients with additional benefits[91]. In extension to this phase III clinical trial, a tissue biomarker study concluded that increased EGFR gene copy number led to worse overall survival and PFS[92].

Table 1 HER-targeted TKIs evaluated in preclinical models of ovarian cancer

TKI	Chemical structure	Pharmacology	Clinical status	IC_{50}: EGFR (nM)	IC_{50}: HER2 (nM)	IC_{50}: others (nM)	Reference
Afatinib (BIBW2992)		Potent and irreversible inhibitor of EGFR/HER2 including erlotinib-resistant EGFR T790 M[71,72]	Marketed for EGFR mutation positive lung cancer	0.5	14	-	[73]
Canertinib (CI-1033)		Irreversible non-selective EGFR family inhibitor, with an additional benefit of blocking mutant EGFRvIII[74]	Reached phase II; discontinued lately by Pfizer	1.8	11	HER4: 27	[75]
Dacomitinib (PF00299804)		Irreversible pan-HER inhibitor, especially EGFR	Phase III clinical trials	6.0	45.7	HER4: 73.7	[75]
Erlotinib (OSI-774)		Specific and reversible inhibitor of EGFR	Marketed for NCSLC and pancreatic cancer	0.5	512	HER4: 790	[75]
Gefitinib (ZD-1839)		Specific and reversible inhibitor of EGFR	Marketed for NCSLC	3.1	343	HER4: 476	[75]
Lapatinib (GW-572016)		Reversible and specific inhibitor to EGFR and HER2[76]	Marketed for HER2 overexpressing breast cancer	10.8	9.2	HER3: 13, HER4: 367	[77,78]
Neratinib (HKI-272)		Potent irreversible, pan-HER (ie, HER 1, 2, and 4) TKI, with low molecular weight[79]	Marketed for adjuvant treatment of HER2 overexpressing breast cancer	92	59	KDR: 800, src: 1400	[80]
Sapitinib (AZD-8931)		Equipotent inhibitor of EGFR, HER2 and HER3[78]	Phase II clinical trials	4	3	HER3: 4	[78]

Lapatinib

Lapatinib (Tyverb®, GW-572016) is an oral competitive TKI inhibitor selective for EGFR and HER2. It is recommended in HER2 overexpressing breast cancer. Preclinical data indicates that lapatinib is effective when HER2 is overexpressed and most likely homodimerized[78]. In breast cancer clinical trials, lapatinib has shown better inhibition of tumors expressing HER2 instead of EGFR[78,93]. In a phase I study in ovarian cancer, while assessing lapatinib in combination with carboplatin many non-dose limiting toxicities were noticed and 6 out of 11 patients had PR or stable disease[94], while in a phase II trial (LapTop) assessing lapatinib with topotecan, only 20% of patients experienced benefit. However, considerable hematologic adverse effects were observed in this trial[95,96]. In another phase II study in recurrent ovarian cancer, the median PFS was 1.8 months, OS was 10.5 months, and only 2 out of 25 patients had PFS at 6 months, while there was no ORR, which might be due to low EGFR and HER2 expression[97].

Sapitinib

Sapitinib (AZD 8931) is a type I, reversible, equipotent inhibitor of EGFR, HER2 and HER3 receptor signaling, especially when EGFR is highly expressed and there is no HER2 overexpression[78,98]. In fact, sapitinib has shown enhanced tumor growth inhibition against EGFR-driven xenograft tumors when compared to lapatinib[78]. Preclinical

studies suggest that sapitinib favors HRG-induced HER2/HER3 heterodimers[78]. It has also demonstrated its ability to inhibit proliferation through pERK and pAkt pathways, and induce apoptosis through M30 and cleaved caspase-3[78]. In xenograft models of inflammatory breast cancer, sapitinib alone significantly inhibited tumor growth, however, the combination of paclitaxel and sapitinib was more effective than either agent alone[98]. Sapitinib has been tested in 8 ovarian cancer patients amongst others, in a phase I clinical trial, to assess the maximum tolerated dose, which was established to be 240 mg twice, daily[99].

Irreversible inhibitors

Afatinib

Afatinib (Gilotrif®, Giotrif®, BIBW-2992) is a type I anilinoquinazoline derivative TKI inhibitor, which irreversibly binds to EGFR, HER2 and HER4[100]. It is currently approved for the treatment of mutated EGFR non-small cell lung cancer (NSCLC). Afatinib has not been extensively tested in ovarian cancer clinical trials. A phase I dose escalation study, which included four ovarian cancer patients, showed promise clinically as indicated by stable disease[101,102]. In breast cancer, afatinib treatment was effective, however, it produced many unwanted side effects in patients[103,104]. In an ovarian cancer preclinical study, afatinib reversed the ATP binding cassette (ABC) mediated multidrug resistance to paclitaxel and adriamycin and also increased the apoptotic efficacy of paclitaxel in ABCB1 overexpressing tumors[105]. In breast cancer, it was also found that afatinib overcomes HER^{T798I}-mediated neratinib resistance[106]. *In vivo*, afatinib with docetaxel showed better response in tumor size reduction, than either drug as a single agent[107]. *In vitro* studies using ovarian cancer cell line models showed that afatinib is effective in inhibiting migration and proliferation[108]. It was also found to be effective in inhibiting basal and heregulin-induced EGFR, HER2, Akt and ERK phosphorylation[108].

Canertinib

Canertinib (CI-1033, PD-183805) is a 4-anilinoquinazoline, irreversible, pan-HER TKI, which reached phase II clinical trials. However, it was recently withdrawn[109]. *In vivo*, canertinib showed potent inhibitory effects in ovarian cancer cell lines, especially when combined with a c-MET inhibitor (PHA665752), which further reduced phosphorylation and total expression of signaling proteins[108,110,111]. A randomized phase II clinical trial in platinum resistant or refractory ovarian cancer patients resulted in disease stability in about 30% of the patients and a one-year survival rate of around 37%. However, there were no complete or partial responses[112]. Studies suggest that high levels of HER and low levels of HER autocrine ligands lead to canertinib resistance[113].

Neratinib

Neratinib (Nerlynx®, HKI-272) is an oral, irreversible pan-HER inhibitor, which has been recently approved for the adjuvant treatment of early stage HER2-positive breast cancer[114], after a phase III study indicated a 2-year, invasive, disease-free survival rate of 94%, when administered after chemotherapy and trastuzumab adjuvant therapy[115]. In a phase II study assessing neratinib in advanced NSCLC, patients with T790M EGFR mutation did not respond to therapy, however, partial response or disease stabilization was seen in patients with G719X mutated EGFR[116]. Overall, previously treated patients or TKI-naïve patients did not benefit notably from neratinib treatment. One of the reasons for this could be low bioavailability due to dose reductions prompted by toxicity[116]. In HER2-positive breast cancer, neratinib as a single agent was well tolerated and has shown substantial clinical activity in trastuzumab-naïve patients, with a 16-week PFS rate of 78% and median PFS of 39.6 weeks versus 16-week PFS rate of 59% and median PFS of 22.3 weeks in trastuzumab pre-treated patients[79]. Other clinical trials in breast cancer assessed neratinib in combination with temsirolimus, vinorelbine, paclitaxel with or without trastuzumab and capecitabine, all of which were well tolerated by patients and had anti-tumor properties[117-121]. Neratinib has demonstrated pre-clinical efficacy in ovarian cancer, especially in HER2-amplified carcinosarcoma, where it inhibits proliferation and tumor growth[122], as well as decreases phosphorylation of transcription factor S6 and causes cell cycle arrest in the G0/G1 phase[123]. There is currently a phase II clinical trial assessing neratinib efficacy in HER2-positive solid tumors (SUMMIT Trial) with mutations in EGFR, HER2 or HER3, including ovarian cancer (NCT01953926). This trial included four evaluable ovarian cancer patients of whom one had stable disease, while three had disease progression[124].

Other HER-targeted TKIs investigated in clinical trials

Dacomitinib (PF00299804), a recently developed irreversible pan-HER receptor inhibitor, has demonstrated interesting anti-proliferative activity against chemoresistant ovarian cancer cell lines[125]. One ovarian cancer patient showed response in a phase I clinical trial of dacomitinib in combination with anti-IGFIR antibody figitumumab[126]. Several multi-targeted TKIs with broad-spectrum activity including the HER family are undergoing evaluation. Vandetanib (ZD6474) is a drug that inhibits EGFR, VEGF receptor and Ret signaling and has been tested as monotherapy in an ovarian cancer trial where, despite decreasing EGFR phosphorylation, it demonstrated little efficacy[127]. Leflunomide is an inhibitor of EGFR, PDGFR, and FGFR and in two phase II trials, 1 out of 8 (12.5%) and 1 out of 15 (7%) ovarian cancer patients demonstrated partial response[128,129].

Biomarkers of sensitivity and resistance to TKIs in ovarian cancer

In breast and lung cancers, informative biomarkers of sensitivity to HER TKIs include overexpression of HER2 and mutation of EGFR. In breast cancer, HER2 overexpression is an effective biomarker of sensitivity to HER2-targeted TKIs, such as lapatinib. Preclinical studies of T-DM1 in ovarian cancers suggest that minimal expression of HER2 was essential for anti-tumorigenic properties of T-DM1 in model systems[66]. Analysis of a series of ovarian cancer xenograft models demonstrated the curative potential of trastuzumab/pertuzumab combination in cancers with amplification and overexpression of HER2. Currently there is less information available regarding the association of HER2 expression levels and TKIs, in ovarian cancer[59,60]. Interestingly, there is one case report of dramatic remission of a chemotherapy-resistant ovarian cancer to trastuzumab, which was HER2-negative suggesting that, the factors governing responsiveness in ovarian cancer might differ from those in breast cancer[130]. The mutated form of EGFR with deletions in exon 19 indicates sensitivity to TKIs such as erlotinib and gefitinib, in NSCLC[131]. However, the importance of EGFR mutations in ovarian cancer is still not well researched since the occurrence of these mutations is much lower. As mentioned above, in a phase II trial of gefitinib in 27 ovarian cancer patients, the single patient showing response did contain an EGFR mutation (2235del15; E746-A750del) in the catalytic domain consistent with this molecular feature being an indicator of sensitivity[82]. This requires further validation in future studies.

The importance of mutations in HER2 and HER3 for sensitivity to pan-HER inhibitors, is under clinical investigation at present and a basket trial (SUMMIT) investigating neratinib treatment in multiple cancers with mutations, has been reported[124].

As observed with other chemotherapeutic drugs, resistance to TKIs is inevitable[132]. Mechanisms of resistance to EGFR-specific TKIs can include abnormalities in HERs, such as HER2 overexpression and mutations like EGFRvIII and HER2^{L869R}[106,133] and secondary EGFR mutations in T790M[134], L747S, D761Y, and T854A[135]. Downstream signaling pathways that are frequently modified include mutations in KRAS, BRAF, PIK3CA, and PTEN[136-138]. Alternative pathways that can bypass control include aberrant activation of MET and HGF[139,140], modifications in VEGF receptors which trigger vascular permeability, in platelet-derived growth factors that regulate angiogenesis and in interleukin-6 that controls inflammatory processes[132-134]. For other TKIs, overexpression of ABC resulting in low drug concentration in cells due to decreased uptake and increased efflux of the drug[141] also contribute in resistance to therapy.

Future research

Combination therapy

Research in various cancers show that the combination of TKIs with chemotherapy, radiation, or mABs significantly inhibits tumor growth, without additional toxic effects, since they have different inhibitory profiles. For instance, lapatinib is administered in combination with either capecitabine/trastuzumab/aromatase inhibitor in HER2-positive breast cancers. Synergistic drug combinations can be achieved in two ways: vertically, which involves similar doses as monotherapy, and horizontally, in which the concentration of the dose is decreased downwards[142]. To date, in ovarian cancer, there are no established combination strategies involving TKIs. In ovarian cancer, only the mAB bevacizumab is approved to be used in combination with paclitaxel and carboplatin. In a phase II ovarian cancer study, the combination of pertuzumab and gemcitabine showed improved overall PFS when compared to gemcitabine and placebo[57]. In breast cancer, the combination of pertuzumab and trastuzumab has shown significant advantages over monotherapy. However, the combination of these mABs in ovarian cancer has only been studied *in vivo*, which has shown promising results[59], and thus might be worth looking at clinically. Additionally, the combination of mABs and TKIs has not been clinically studied so far, in ovarian cancer. The combination of HER-targeted inhibitors with Poly (ADP-ribose) polymerase (PARP) inhibitors might prove useful in the treatment of ovarian cancer, since, recent *in vitro* studies combining a TKI with a PARPi showed synergistic growth inhibitory effects[143]. Computational

biology has developed in recent years and offers the potential of precision medicine. Molecular anomalies can now be detected when screening through molecular information, which could identify individual patient appropriate medication . Hence, more pre-clinical bioinformatics studies need be conducted to investigate the effects of therapy on a molecular basis.

Chemosensitive versus chemoresistant phenotypes

HGSOC usually responds well to initial ovarian cancer therapy, with response rates as high as 85%[7]. This is frequently due to the fact that HGSOC has BRCA mutations. First-line chemotherapy acts by damaging DNA strands; BRCA acts to repair DNA, however, given that HGSOC frequently lacks BRCA function, DNA strands cannot be repaired, which consequently leads to the efficacy of primary chemotherapy in HGSOC. However, HGSOC cells through further mutation can restore BRCA function, which is one of the leading causes of resistance to initial chemotherapy[144].

It is often acknowledged that ABC transporters play a pivotal role in resistance to first line chemotherapy. In fact, *ABCB1*, *ABCB4*, and *ABCG2* were significantly up-regulated in cisplatin and paclitaxel resistant ovarian cancer cells[145]. Pertuzumab in a clinical trial of chemoresistant disease showed that the mAB demonstrated possible anti-tumor activity when combined with either gemcitabine or paclitaxel[57,58], while novel TKIs that target multiple sites often demonstrate their ability to reverse ABC-mediated drug resistance[105,146-148]. Hence, mABs and TKIs might be more active in chemoresistant ovarian cancer than in the chemosensitive type.

Conclusions

Ovarian cancer is a complex disease, with multiple molecular profiles. It frequently becomes resistant after initial therapy necessitating the development of new strategies.

The use of HER-targeted therapy continues to be assessed in this disease, since it might have value for selective patients and pre-clinical data supports the potential of this approach. Only a limited number of phase II trials have been completed in ovarian cancer and while response rates are low, there are frequent good percentages of stable disease. The pan-HER TKIs may have broader efficacy and utility than the early EGFR-targeted TKIs, which are dependent on the presence of mutations (are uncommon in ovarian cancer). Further biomarker studies are now required to help identify the most sensitive ovarian cancers and combination strategies require further development.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

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